THE ROLE OF METAL CATIONS IN THE CONTROL OF *IN VIVO* CHLOROPHYLL *a* FLUORESCENCE

by

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ABSTRACT

A study has been made of the influence of inorganic cations on *in vivo* chlorophyll fluorescence both with isolated intact and broken chloroplasts. It has been shown that changes in chlorophyll fluorescence brought about by varying the organic or inorganic cation content of the suspending medium of broken chloroplasts reflects the interaction of cations with fixed negative charges on the *outer* surface of the thylakoid membrane. The results can be quantitatively interpreted in terms of Gouy-Chapman double layer theory and are consistent with the existence of an average fixed negative charge density of 2.5 $\mu C\ cm^{-2}$ on the outer surface of the thylakoid membrane. In the isolated intact chloroplast and probably in the leaf, stromal levels of metal cations, mainly Mg^{2+}, appear to be sufficient to maintain *in vivo* thylakoids in a high fluorescing conformational state. Lowering of fluorescence from the high yield also occurs on initiating light induced electron transport and associated proton pumping and seems to be due to protonation of fixed negative charges on the *inner* surface of the thylakoid membrane. Evidence is presented that fluorescence changes associated with events at both the inner and outer thylakoid surfaces monitor changes in the relative distribution of light energy to photosystem I (PSI) and photosystem II (PSII). However, in the intact chloroplast it seems likely that the magnitude of the light induced transthylakoid pH gradient is the main factor controlling chlorophyll fluorescence and may be important in maintaining an overall maximum utilisation of the incoming light quanta driving photosynthesis.

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SYMBOLS AND ABBREVIATIONS

A	Secondary electron acceptor of PSII reaction centre, or = $(RT\varepsilon/2\pi)^{\frac{1}{2}}$
9-AA	9-aminoacridene
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
C	Coulomb
$C_{\frac{1}{2}}$	Concentration of reagent required to induce half the maximal effect
C-550	Absorption change at 550 nm
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
Chl678	Chlorophyll a having an absorption maximum at 678 nm
$Chla_I$	Chlorophyll reaction centre pigment of PSI
$Chla_{II}$	Chlorophyll reaction centre pigment of PSII
Cyt	Cytochrome
c or c_∞	Concentration of reagent in bulk solution
c_o	Local concentration of cations in solution adjacent to the thylakoid surface
DAD	2,3,5,6-tetramethyl-p-phenylenediamine (diaminodurene)
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6 dichlorophenolindophenol
$DCPIPH_2$	Reduced DCPIP
DEAE-cellulose	Diethylaminoethyl cellulose
DLE	Delayed light emission
dc	Actinic light, non-modulated
EDTA	Ethylenediaminetetra-acetic acid
F685, F695, F730	Fluorescence emission at 685, 695 or 730 nm
F1	Fluorescence emission from chlorophylls of PSI
F_o	Constant yield of chlorophyll a fluorescence

FeCy	Potassium ferricyanide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
I	Light intensity
I_f	Intensity of fluorescence emission
I_o	Intensity of absorbed light
K_f	Rate constant for fluorescence
K_h	Rate constant for radiationless deexcitation
K_p	Rate constant for trapping at an open PSII reaction centre
K_t	Rate constant for transfer to chlorophyll of PSI
MeVi	Methyl viologen
m	Modulated light
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced $NADP^+$
nig	Nigericin
OAA	Oxaloacetic acid
OIDPSMT	Transients in the induction of chlorophyll a fluoresence in vivo
P680	Absorption maximum of $Chla_{II}$
P700	Absorption maximum of $Chla_{I}$
Pi	Inorganic phosphate
PGA	Sodium 3-phosphoglycerate
PMS	Phenazine methosulphate
PPNR	Photosynthetic pyridine nucleotide reductase
PSI	Photosystem I
PSII	Photosystem II
PSU	Photosynthetic unit
p	Probability of transfer of excitation energy between PSII photosynthetic units
Q	Primary electron acceptor of PSII reaction centre
q	Fixed net negative charge density on the thylakoid surface

RuDP	Ribulose 1,5 diphosphate
tris	Trishydroxymethylaminomethane
X	Primary electron acceptor of PSI reaction centre
Y	Secondary electron donor to PSII reaction centre
Z	Oxygen evolving (water splitting) enzyme of PSII
α	Fraction of light absorbed or transferred to chlorophyll a of PSII
β	Dimensionless constant
ΔpH	Transthylakoid pH gradient
$\Delta\psi$	Transthylakoid electrical gradient
ε	Permittivity of water
ε_o	Permittivity of free space (vacuum)
ρ	Average space charge density of ions in solution
τ	Lifetime of an excited state
τ_o	Natural (intrinsic) lifetime of an excited state
ϕ_f	Quantum yield of variable chlorophyll a fluorescence
ϕ_p	Average quantum yield of photochemical trapping in PSII
ψ	Electrical potential difference relative to the bulk solution
ψ_o	Electrical potential difference at the thylakoid surface relative to the bulk solution

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SECTION 1

INTRODUCTION

1.1. Some Concepts of Photosynthesis

1.1.1. General Introduction

The way in which solar energy is trapped and converted into chemical energy by chlorophyll-containing organisms is termed photosynthesis. In nature, the only plentiful continuous source of energy is sunlight. Consequently, virtually all forms of life are ultimately dependent on the complex organic molecules synthesised by photosynthetic organisms, both as a source of biological energy, and for the various processes involved in growth and the maintenance of life. For this reason alone, the importance of a thorough knowledge of the mechanism by which photosynthetic energy transduction occurs can hardly be denied.

Moreover, the continuing depletion of fossilised fuel reserves has, in recent years, caused an increasing amount of attention to be paid to the vast, untapped potential of sunlight as a direct, alternative source of energy. There would be little of the present concern for our future energy needs if man-made ways could be devised of trapping solar energy in such a useful and efficient way as that which occurs during photosynthesis.

In more academic terms, photosynthesis offers a field of research in which workers from many branches of science can participate. Because no one person can hope to master all the various principles developed in physics, chemistry and biology, the presentation and interpretation of experimental results in much of the published literature of photosynthesis has been of necessity descriptive in nature. To be fair, this is partly because we can rarely hope to control with any precision the numerous and often poorly understood experimental variables inherent in the study

of complex biological systems. However, our understanding of photosynthesis in molecular terms must ultimately involve application of firm physico-chemical principles to what at present is an often hazy, and ill-defined qualitative picture. The cooperation of physicists, chemists and biologists is vital in fulfilling what is the basic aim of all biological research, to describe the process in molecular terms. This is particularly true for research in photosynthesis.

I hope to illustrate some of the points made here in the course of this thesis, where an attempt will be made to gain a better understanding of the way in which metal cations may be used by green plants to help regulate the overall efficiency of photosynthesis *in vivo*.

1.1.2. Photosynthesis: dark and light reactions

Apart from bacteria which will not be considered in this thesis, most photosynthetic organisms utilise water as an electron (or hydrogen) donor to reduce carbon dioxide to the level of carbohydrates. The overall process, represented in a simplified way in equation 1.1., is thermodynamically unfavourable as written, and energy in the form of light must be supplied to drive electrons from the oxido-reduction potential of H_2O/O_2 couple to that of CO_2/O_2.

$$nH_2O + nCO_2 \xrightarrow{\text{light}} (CH_2O)_n + nO_2 \qquad \text{Eq. 1.1.}$$

In fact the oxidation of water is separated from the reduction of CO_2 by a host of intermediate steps which have, for experimental convenience rather than sound theoretical reasons, been divided into so-called 'dark' and 'light' reactions.

The 'dark' reactions constitute a complex series of non-photochemical, enzyme-mediated biochemical steps which use ATP and NADPH to reduce CO_2 to the level of sugars. First mapped out by Calvin and coworkers[1] using

radioactively labelled CO_2, these reactions are known as the Calvin cycle, and under certain conditions,[2] can occur without the need of light providing ATP and NADPH are supplied.

In the living organism, ATP and NADPH are normally supplied to the Calvin cycle by the 'light' reactions. In 1937,[3] Hill first showed that an illuminated cell-free preparation of chloroplasts (see Section 1.1.2) could reduce other compounds (notably ferric salts) but not CO_2, with concomitant O_2 evolution. The light-dependent reduction of artificial electron acceptors by isolated chloroplasts linked to the production of O_2 became known as the Hill reaction, and a number of such electron acceptors were discovered including $NADP^+$.[4]

In 1954, Arnon et al discovered the process of photosynthetic phosphorylation.[5] They found that illumination of isolated chloroplasts resulted in the formation of ATP from ADP and inorganic phosphate (Pi), and later[6] that this was somehow associated with light dependent reduction of $NADP^+$. The reduction of $NADP^+$ and associated ATP production are generally considered to constitute the end-point activity of the 'light' reactions, whereas the use of these metabolites in CO_2 fixation represents the 'dark' reactions. As in any metabolic pathway, however, such a division is somewhat artificial and ignores the subtleties involved in regulation and metabolic control mechanisms. The interplay between the light and dark reactions involves considerably more than the production and consumption of ATP and NADPH. However, consideration of such matters must be deferred until some basic concepts of the way photosynthesis is thought to operate have been introduced.

1.1.3. Structure and compartmentation of the chloroplast

The whole process of photosynthesis occurs in semi-autonomous organelles within the cell of higher plants, termed chloroplasts.[7,8]

Furthermore the intact chloroplast is compartmentalised, and the light and dark reactions localised in different regions. Figure 1.1. shows an electron micrograph of an intact chloroplast from spinach. It can seen that the organelle is surrounded by a double outer membrane called the envelope. The outer of these two membranes is permeable to a number of low molecular-weight substances.[9,10] The innermost membrane of the envelope appears to act as an osmotic barrier between the internal chloroplast space and the external medium. Thus the intact chloroplast has been shown to be impermeable to simple-diffusion controlled penetration of metal cations, sugars, phosphorylated sugars and nucleotides, inorganic phosphate and dicarboxylic and tricarboxylic acids.[9-12] Carbon dioxide appears to be freely permeable as the dissolved gas.[9] Complex traffic of metabolites between chloroplast and cytoplasm does, however, occur across the outer envelope mediated by specific translocators. Three types of translocator have so far been recognised,[9,10] allowing exchange across the envelope of (i) phosphorylated triose sugars and inorganic phosphate; (ii) adenine nucleotides especially ATP, and (iii) dicarboxylic acids. No direct exchange mechanism has yet been discovered for $NADP^+$/NADPH.

The outer envelope does not carry out any of the light or dark reactions directly concerned with photosynthesis. Its major role appears to be to maintain and regulate the internal environment of the chloroplast as will be extensively discussed throughout this work.

Found within the envelope is a second, inner-membrane system in which is embedded the machinery of the primary, or light reactions. The inner membrane system (thylakoids) consist of enclosed lamellar sacs, and thus divide the internal chloroplast space into two compartments. The intra-thylakoid space apparently contains little in the way of soluble photosynthetic apparatus, whereas the space between the thylakoids and the outer membrane, termed the stroma, is a matrix of 'soluble' enzymes

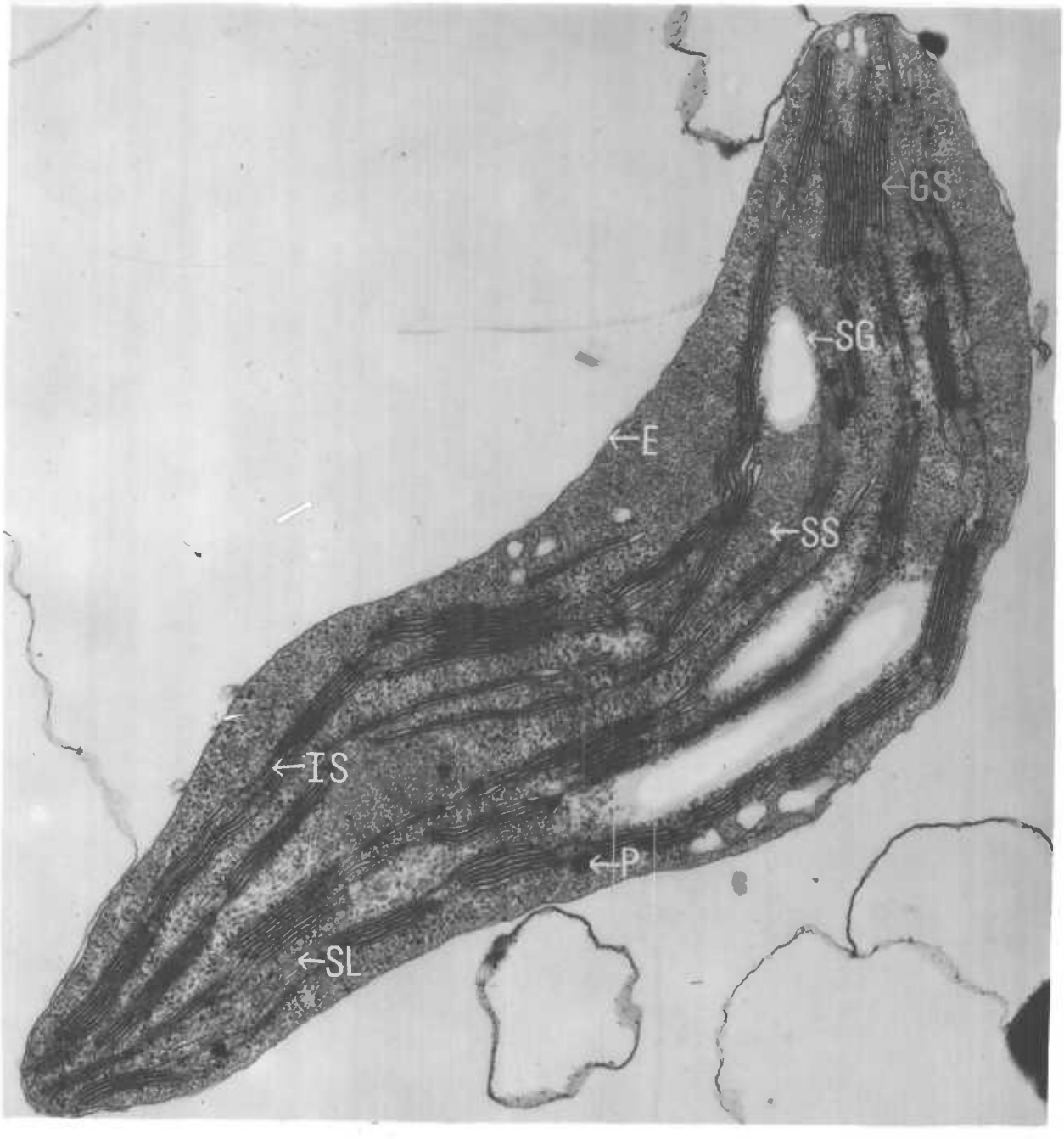


Figure 1.1 Electron micrograph of an intact spinach chloroplast suspended in assay medium. E outer envelope; SS stromal space; SG starch granule; SL stromal lamellae; GS grana stack; IS intra-thylakoid space; P osmiophylic particle (courteousy of Dr A. Telfer, see Ref 192 for details of fixation and staining procedure).

including those of the dark reactions (Calvin cycle). In many plants, a large proportion of the inner lamella system occurs in the form of appressed discs of approximately 5,000 Å diameter and 150-300 Å thickness.[13] These are termed grana lamellae, and are found stacked together in the form of piles of coins. The number of granal discs in a stack varies between species, but in spinach can be of the order of a dozen or more. Other regions of the thylakoid membrane apparently do not stack in vivo and have been termed stromal lamellae. Stacking appears to be caused by the high stromal level of cations as discussed in Section 1.4.2.

The reason why some regions of the thylakoid membrane occur as stacked grana, and others as unstacked stromal lamellae is not clear. Evidence obtained from separation of these two types of membrane by mechanical disruption and differential centrifugation of chloroplasts indicates that grana possess the full capacity of the light reactions whereas stromal lamellae contain only photosystem I (see Section 1.1.4) and lack other features.[7,14]

Comparison of chloroplasts obtained from certain tropical grasses shows that stacking of thylakoids which occurs in some tissues, but not in others, is related to the specialisation of these tissues for a particular role in overall carbon metabolism.[7,15] Again, stacked membrane configuration appear in chloroplasts most completely equipped to carry out both the light and dark reactions of CO_2 fixation.

Carefully prepared[16,17] isolated chloroplasts which retain their outer envelopes undamaged are able to photosynthetically fix CO_2 at rates comparable to those in the living organism. However, the outer envelope is somewhat fragile and unless special precautions are taken, is easily damaged or removed during chloroplast preparation, and part or all of the contents of the stroma lost to the isolation medium.[18] Such 'broken chloroplasts' show little or no ability to fix CO_2, but can carry

out the light reactions of photosynthesis providing that artificial electron acceptors are added in substrate amounts (see, for instance Ref.5).

The structural aspects of chloroplasts have been stressed here for several reasons. Firstly, although chloroplasts without outer envelopes have been of great use (see Ref.19) in elucidating the mechanisms of electron-transport mediated $NADP^+$ reduction and O_2 evolution and phosphorylation (to be discussed shortly), the loss of the stroma in these preparations has inevitably meant that studies have been carried out using non-physiological chloroplast suspending media. There is little doubt that different and sometimes conflicting results obtained by different workers in the past on photosynthetic aspects to be mentioned, have sometimes been due to different suspending media used (see reviews in Ref.8). This must be born in mind when relating results obtained using broken chloroplasts in experimentally determined conditions to those obtained under conditions prevailing in the living organism.

Secondly, study of intact chloroplasts capable of CO_2 fixation offers an intermediate and experimentally amenable stage between studies of broken chloroplasts which do not fix CO_2 and the living organism which is often difficult to handle experimentally.

Thirdly, the intact chloroplast, both in the cell (in vivo), and isolated so as to retain the outer envelope, is a two-compartment system, and as such may be subject to control processes brought about by a change in the partition of ions or metabolites between compartments. Such control processes may not be seen in the broken chloroplast system.

With these points in mind, I will review in the following sections how current ideas on the mechanism of the light reactions (see Sections 1.1.4. and 1.1.5) may lead to the generation of ion-fluxes in the intact chloroplast (see Section 1.1.6.), and how these fluxes may affect CO_2

fixation (see Section 1.1.7) and the primary light reactions of photosynthesis themselves (see Section 1.2. and 1.3)

1.1.4. Two light reactions and the formulation of the Z-scheme

All oxygen evolving organisms contain chlorophyll a (which exists in several different forms in vivo)[20] as a major proportion of the light absorbing pigments, together with a number of auxiliary pigments including chlorophyll b (green plants), phycobilins (red and blue green algae) and a variety of carotenoids. Using short flashes of light to excite photosynthesis, Emerson and Arnold[21] estimated that in chlorella approximately 2,500 chlorophyll a molecules cooperate in the production of one O_2 molecule. This gave rise to the concept that only a small proportion of chlorophyll a is involved in the production of chemical energy from light (trapping) and that most of the chlorophyll present serves only to absorb light and deliver the excitation energy to the trapping centres (or reaction centres). Duysens[22] later showed that chlorophyll fluorescence could be excited by light absorbed by the auxiliary pigments, indicating that these pigments also transfer absorbed excitation energy to the reaction centres via chlorophyll a.

The reaction centre and its associated light harvesting pigments is termed a photosynthetic unit, and in this sense is purely a statistical notion. Since it is now believed that eight quanta are utilised in the production of one oxygen molecule, the average number of chlorophyll molecules associated with each reaction centre from the data of Emerson and Arnold would be 300.

Later work of Emerson and co-workers led to the idea that there were two kinds of photosynthetic unit characterised by different absorption spectra.[23,24] Working with algae, it was observed that the quantum yield for O_2 evolution markedly decreased at wavelengths of monochromatic light longer than 690 nm, a region where chlorophyll a absorption is

still prominent.[23] Furthermore, this 'red-drop' in the quantum yield seen in the far-red region of the *in vivo* absorption spectrum was not observed when simultaneously, a second monochromatic light of shorter wavelength was applied.[24]

This effect, known as 'enhancement', led Emerson and Rabinowitch to propose[25] that *in vivo* two different forms of chlorophyll *a* sensitised two different light reactions.

Modern ideas[26,27] of the detailed mechanism of $NADP^+$ reduction linked to oxygen evolution incorporate these earlier observations with the electron transport scheme proposed by Hill and Bendall.[28] Current models, one of which is depicted in Fig.1.2 vary somewhat in details, but generally propose that not one, but two photo acts (connected by a series of electron carriers) mediate the reduction of $NADP^+$ by electrons derived from water. This arrangement is popularly known as the Z-scheme of electron transport.

The two light reactions are generally proposed to be sensitised by discrete pigment systems, or photosystems, each of which contain photosynthetic units characteristic of photosystem I (PSI) or photosystem II (PSII). Thus photosystem I is proposed to contain the long wavelength forms of chlorophyll *a*, whilst PSII contains relatively more of the shorter wavelength absorbing chlorophyll *a* and proportionately more of the auxiliary pigments.[27]

A quantum of light absorbed anywhere within a PSII unit raises the absorbing pigment to the excited singlet state. The exciton is then transferred rapidly to the reaction centre (probably by a Förster-type resonance energy transfer mechanism, see Ref.29), where it is trapped and converted into chemical energy. A PSII reaction centre is thought to be composed of a specialised chlorophyll *a* molecule, $chla_{II}$ (or a dimer[30]) in close association with the primary electron acceptor Q. In turn, $chla_{II}$ and Q are associated with a secondary donor and acceptor,

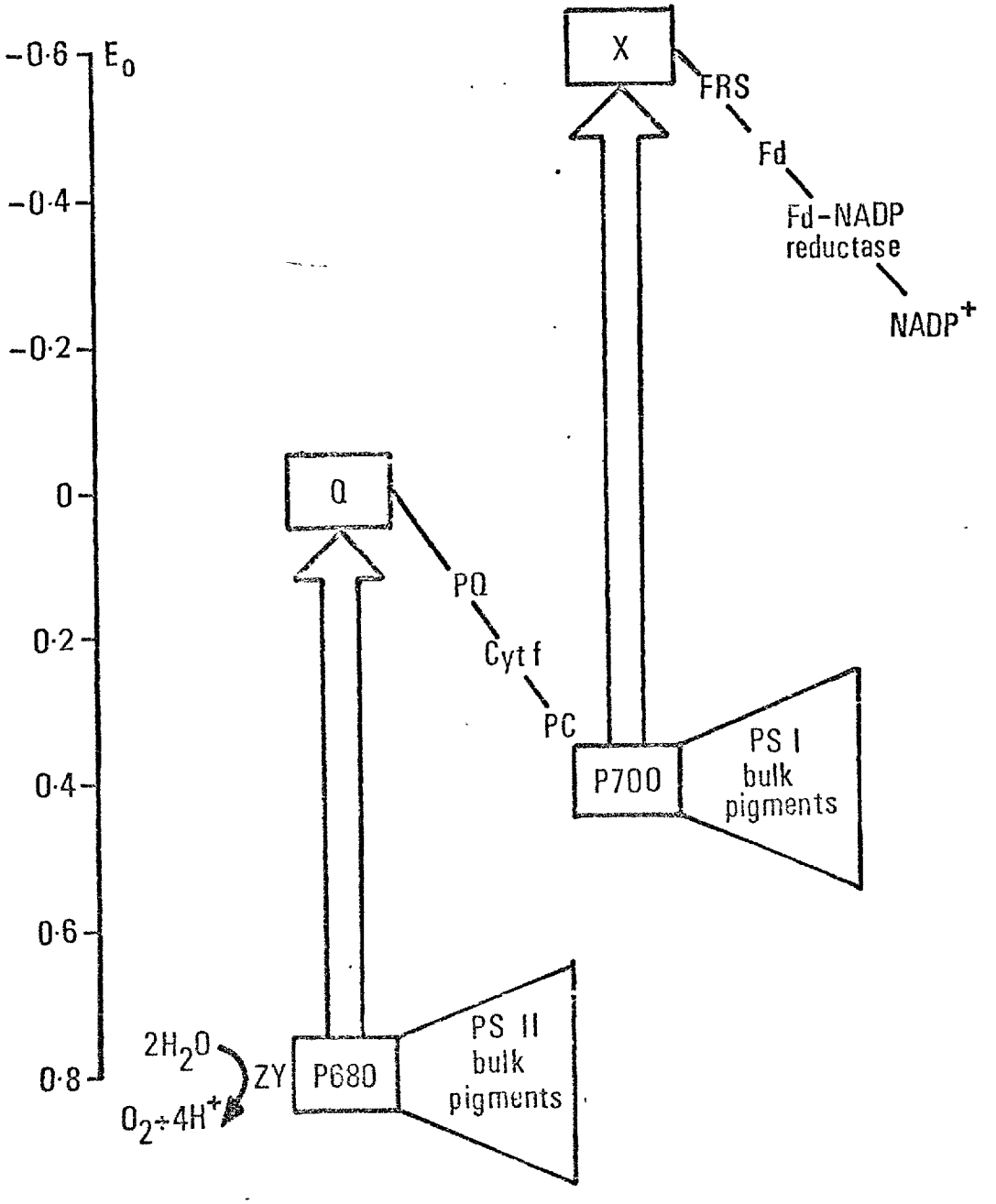


Fig. 1.2 A current version of the Z-scheme of photosynthetic electron transport. Electrons are raised from the redox potential of the H_2O/O_2 couple to that of NADPH/NADP+ via two light reactions mediated by photosystem I(PSI) and photosystem II(PSII). The two photosystems are connected by a series of electron carriers: plastoquinone(PQ), cytochrome f(Cyt f), and plastocyanin(PC), by which the primary electron acceptor of PSII (Q) may reduce the primary donor of PSI (P700). The reduction of NADP+ by the primary electron acceptor(X) of PSI is mediated by 'ferredoxin reducing substance(FRS), ferredoxin (Fd) and ferredoxin-NADP+ reductase. Oxidation of H_2O by the primary electron donor of PSII is mediated by the components Y and Z.

Y and A respectively.[31]

$$Y\ (Chla_{-II}\ Q)\ A \xrightarrow{h\nu} Y\ (Chla_{-II}^{*}\ Q)\ A \rightarrow Y\ (Chla_{-II}^{+}\ Q^{-})A. \qquad \text{Eq. 1.2}$$

Equation 1.2 represents the trapping process when a $Chla_{-II}$ is excited on receipt of excitation energy from a light harvesting pigment (not shown). The metastable state $Y(Chla_{-II}^{+}\ Q^{-})A$ is known as a charge transfer complex and represents the first production of electrochemical energy from absorbed light. The oxidation of $Chla_{-II}$ results in a small absorption decrease of 682 nm[32,33] thought to reflect the bleaching of the reaction centre chlorophyll absorption band. The rise time (less than 20 nsecs) of this absorption change (termed P680) is, as yet, too fast to resolve by conventional techniques. However, the advent of picosecond, optical measurements should soon allow complete kinetic analysis of the trapping process in chloroplasts.[34] If the oxidation of P680 does reflect primary trapping of excitation energy, one would certainly expect rapid kinetics. Both transfer of the excitation energy to the reaction centre and trapping itself must occur well within the natural lifetime of the excited state (approx. 5 nsecs), or the exciton will dissipate by non-useful pathways such as chlorophyll fluorescence[27] (see Section 1.2). After charge separation has occurred, the trap is said to be closed, and cannot accept a second quantum of light until secondary electron flow both reoxidises Q^{-} and reduces $Chla_{-II}^{+}$.[31] Secondary electron flow occurs with following sequence:

$$Y(Chla_{-II}^{+}\ Q^{-})A \rightarrow Y^{+}(Chla_{-II}\ Q^{-})A \rightarrow Y^{+}(Chla_{-II}\ Q)A^{-} \qquad \text{Eq. 1.3.}$$

Y^{+} is a strong oxidant which eventually extracts electrons from water. However, flash experiments have demonstrated that O_2 evolution only occurs after four oxidising equivalents have been stored by the water splitting apparatus.[35,36] The nature of both Y and Z (which may be parts of the

same complex) are as yet poorly understood. It has been suggested that Z contains manganese, and that the four-quantum oxidation of water may involve the raising of manganese to higher oxidation states.[37] As judged from the decay of P680 absorption changes, reduction of $Chla_{II}^{+}$ occurs within the range 1-200 μsec.[32,33] depending on which electron donor on the oxidising side of PSII happens to be rate-limiting.

The nature of the primary electron acceptor, Q, is also uncertain, but has been identified by some workers[21,38] with a plastoquinone moiety in a specialised environment within the thylakoid membrane. The evidence for this comes mainly from the interpretation of a flat absorption band in the ultraviolet region of the spectrum, which shows small differences from that expected of plastoquinone. Re-oxidation of Q^{-} by A (thought to be the remainder of the plastoquinone pool,[31] occurs with a half time of 0.6 msec. which is slower than reduction of $Chla_{II}^{+}$. Because of the difference in kinetics between secondary electron flow on the oxidising and reducing side of PSII, the return of the reaction centre to the open form ($Y^{+}(Chla_{II}\ Q)A^{-}$) in which trapping can again procede, generally follows the relatively slower re-oxidation of primary acceptor, Q^{-}. This, in turn, leads to characteristic changes in chlorophyll fluorescence[39] discussed in Section 1.2.

The reaction centre of a PSI unit is thought to be analogous to that of PSII. Transfer of an exciton absorbed in PSI to the reaction centre results in trapping with the generation of a charge transfer complex $Chla_{I}^{+}\ X^{-}$. Oxidation of the primary electron donor $Chla_{I}$ also results in a small absorption decrease[40] though at slightly longer wavelengths (700-705 nm) than that for PSII. X^{-} is a strong reductant that eventually donates electrons to $NADP^{+}$, whilst $Chla_{I}^{+}$ is a somewhat weaker oxidant than $Chla_{II}^{+}$, and is reduced by Q^{-} via the electron transport chain linking the two photosystems.[26]

The flow of electrons from water to $NADP^+$ mediated by the two photosystems of the Z-scheme is known as non-cyclic electron transport. Such electron transport is associated with ATP formation as discussed in the next Section.

For non-cyclic electron transport to be efficient, equal distribution of light energy to the two photosystems is required. The earlier results of Emerson[23,24] and co-workers suggest that light of wavelengths greater than 690 nm is absorbed proportionately more by PSI while a greater fraction of light of wavelength less than 690 nm is absorbed by PSII. Thus the phenomenum of enhancement observed by these workers is clearly the increased efficiency of electron transport which results from a balancing of light distribution to the two photosystems where a light favouring PSI (PSI light) is combined with one favouring PSII (PSII light).[41]

A great deal of evidence supporting the Z-scheme has accumulated since these classic experiments were reported. It has been found that light thought to favour PSII causes overall reduction of the intermediate electron carriers plastoquinone,[31] cytochrome f[42] and P700,[40] whereas PSI light causes oxidation of these intermediates. Use of specific inhibitors together with artificial electron donors and acceptors has clearly shown the presence of two photosystems by demonstration of the functioning of one photosystem independently of the other.[43-46] Like non-cyclic electron flow through both photosystems partial reactions involving only PSI or PSII can be coupled to ATP synthesis and have been of great use in elucidating the sequence of natural carriers in the Z-scheme[43-46] and the sites associated with ATP synthesis[43,47,48] (see Section 1.1.5).

Under certain conditions both <u>in vivo</u> and in isolated chloroplasts, cycling of electrons around photosystem I seems to occur when the primary

electron acceptor, X, reduces, not the next component in the chain leading to $NADP^+$, but an oxidised component at or before the primary PSI electron donor.[49] Known as cyclic electron flow, this type of electron transport results in no net accumulation of oxidised or reduced products, but is associated with phosphorylation by which it is generally detected.[49] Cyclic phosphorylation can be stimulated in broken chloroplasts by certain artificial co-factors such as PMS[50] and DAD[51] which in the oxidised form can be reduced by PSI and in the reduced form can donate electrons to PSI.

However, evidence exists that phosphorylation linked to cyclic electron flow may also occur in the living cell without the need to add artificial co-factors. This has generally been inferred from the demonstration of light-induced energy-dependent secondary processes under conditions when non-cyclic electron flow is not operating.[49] The pathway of this 'endogenous' cycle electron flow may involve the reduction of plastoquinone[52] by ferredoxin, possibly mediated by cytochrome b564.[53] Although cyclic electron flow around PSI has been proposed to be of physiological significance *in vivo*,[49] there is no evidence for such ATP-linked cyclic electron transport around PSII.

Much qualitative evidence exists to support the concept of the Z-scheme although many questions remain unanswered. The chemical identity of many of the proposed electron carriers in the scheme, particularly on the oxidising side of PSII, has yet to be established.[37] Conversely, the role of other components such as cytochrome b559, thought to be involved in electron transport[54] is yet to be made clear.[26]

More attention needs to be paid to the structural arrangement of the components of the Z-scheme in the thylakoid membrane. Although work in this area is progressing, particularly in relation to the way in which phosphorylation is coupled to electron transport (discussed in the next Section), relatively little is known of the micro-environment in which

the intermediate electron carriers find themselves within the membrane.

The recent isolation of a large number of polypeptides by complete detergent solubilisation and denaturation of the thylakoid membrane[55-57] may indicate that certain components involved in the Z-scheme occur in the form of protein complexes in the membrane. Such complexes may be specifically arranged in the thylakoid so as to improve greatly the efficiency of light capture and electron transport.

As yet, little evidence exists that the intermediate electron carriers are associated with proteins in the chloroplast, but several types of chlorophyll/protein complexes have been isolated using detergent treatments which solubilise, but do not totally denature the thylakoid.[57-60] Three basic types of chlorophyll/protein complex have been identified and characterised. One, containing much of the longer wavelength absorbing forms of chlorophyll a is enriched with the PSI reaction centre and appears to be a PSI photosynthetic unit which has lost part of the outer light harvesting pigment (P700-chlorophyll a complex).[58-60] A second type of complex similarly appears to be part of a PSII photosynthetic unit and contains the shorter wavelength absorbing chlorophyll a together with a PSII reaction centre.[59,60] Both of these sub-chloroplast particles are capable of performing photoreactions characteristic of PSI and PSII respectively.

It is to be hoped that further fractionation of these complexes will allow isolation of active reaction centres of PSII and PSI which are largely free of the associated light harvesting pigments. This should allow greater insight into the little known mechanisms of the primary excitation energy trapping processes.

In addition in chlorophyll b containing organisms, a third sub-chloroplast particle has been isolated (chlorophyll a/b light harvesting pigment).[58] This is also a protein complex and contains chlorophyll a and chlorophyll b in equimolar proportions, but apparently contains no

active reaction centre. It has been proposed that the latter complex may serve purely as a light harvesting pigment system delivering absorbed excitation energy mainly to PSII, but also to a smaller extent, to PSI.[58] This raises the question as to how discreet the pigment systems serving PSII and PSI are in the membrane. Recent work (which will be reviewed in Sections 1.2 and 1.3) has, in fact, shown that the relative distribution between PSII and PSI, of light of a particular wavelength is not immutably fixed, but can vary both *in vivo* and in isolated chloroplasts depending on the particular experimental conditions used.

This leads to the concept that the properties of a photosynthetic unit are not static, but 'dynamic' and may change during illumination as the structure of the membrane undergoes light induced changes (see Section 4.1.).

Such structural changes may well occur in response to light-induced ion fluxes associated with the mechanism of ATP synthesis, discussed in the next two Sections.

1.1.5. Phosphorylation and the vectorial arrangement of the Z-scheme in the thylakoid

The formation of ATP from ADP and Pi by illuminated broken chloroplasts was first discovered by Arnon *et al*[5] and later found to be associated with both cyclic and non-cyclic electron flow.[5,6] Little is known even today of the actual chemistry involved in ATP synthesis, though several hypotheses have been advanced concerning the nature of the coupling between electron transport and phosphorylation. Until 1960, it was generally presumed that energy released during the passage of an electron down the redox potential scale between PSII and PSI was directly linked to ATP formation by the respective formation and utilisation of chemical (covalent) intermediates,[61] as in classical solution chemistry.

However, the introduction by Mitchell in 1961[62] of the chemiosmotic coupling mechanism has led to recognition of the importance of membrane physiology in the role of coupling of electron transport to phosphorylation.

In essence,[62-64] the chemiosmotic hypothesis takes account of the fact that in the course of electron flow from H_2O to $NADP^+$, certain components of the Z-scheme (notably plastoquinone) involve uptake and release of protons during their redox cycle. Since electron transport is a membrane bound process, an assymmetric arrangement of the electron (and proton) carriers in the membrane is proposed, so that protons are always taken up on one side, and released on the other, resulting in a net translocation of H^+ across the membrane stoichiometrically coupled to electron transport.

Providing that the membrane is reasonably impermeable to protons, the energy initially conserved in such a gradient would consist of a chemical concentration term (ΔpH) and an electrical potential ($\Delta\psi$) across the membrane. The total high energy state, or 'proton-motive force' can thus be written:

$$HES = pmf = \frac{RT}{F} \Delta pH + \Delta\psi \qquad \text{Eq. 1.4.}$$

where R is the gas constant, T the absolute temperature and F the Faraday and pmf is in mV.

Such a gradient can be visualised to drive phosphorylation if the ATP synthesising enzyme is also assymmetrically arranged in the thylakoid membrane. Since esterification of ADP with Pi is essentially a dehydration reaction, this would be favoured if the elements of water were released as OH^- and H^+ on the side of the membrane where their concentrations are respectively lowest. The crucial difference between this hypothesis and the earlier chemical hypothesis is that the immediate high energy state precursor of ATP synthesis is an electrochemical proton gradient

rather than an 'energy-rich' covalent bond.

Mitchell's scheme appears to accommodate much of the available evidence as regards phosphorylation and ion movements in chloroplasts[65] and thereby has achieved widespread acceptance.

Electron transport has been shown to result in the generation of transmembrane pH and electrical gradients in broken chloroplasts.[19,31,43-48,50-52,65,66-68,76-82] Data on the electrical potential across the thylakoid stems partly from the study by Witt and co-workers of an electrochromic shift in the absorption spectra (ΔA515) of chloroplasts.[31,65-68] Using brief flashes of light which turnover the photosynthetic apparatus only once, Schliephake *et al*[67] estimated that each charge separation in PSI or PSII results in a transmembrane potential of approximately 25 mV, and that this potential can contribute to ATP synthesis.

Since the rise time of ΔA515 is extremely rapid (less than 20 nsec.) it has been proposed[37,66] that ΔA515 monitors a vectorial arrangement of primary charge separation in the thylakoid membrane. As visualised in Fig. 1.3, this would occur if the primary electron acceptors Q and X of PSII and PSI respectively are located on the outer (stroma facing) side of the thylakoid, and the primary electron donors on the opposite side of the membrane. Use of chemical probes and specific antibody studies has given some support to this, though data is far from conclusive, especially for PSII.[43,69-71]

Other evidence for the vectorial arrangement of the PSII reaction centre in the membrane has been provided from studies of *delayed light emission* (DLE). DLE is thought to arise from de-excitation of chorophyll singlet states created by a direct back reaction of the primary photochemical trapping events at the PSII reaction centre.[72] It has been shown[73,74] that DLE is markedly stimulated on imposing artificially generated electrical potentials (of polarity inside positive) across the thylakoid membrane.

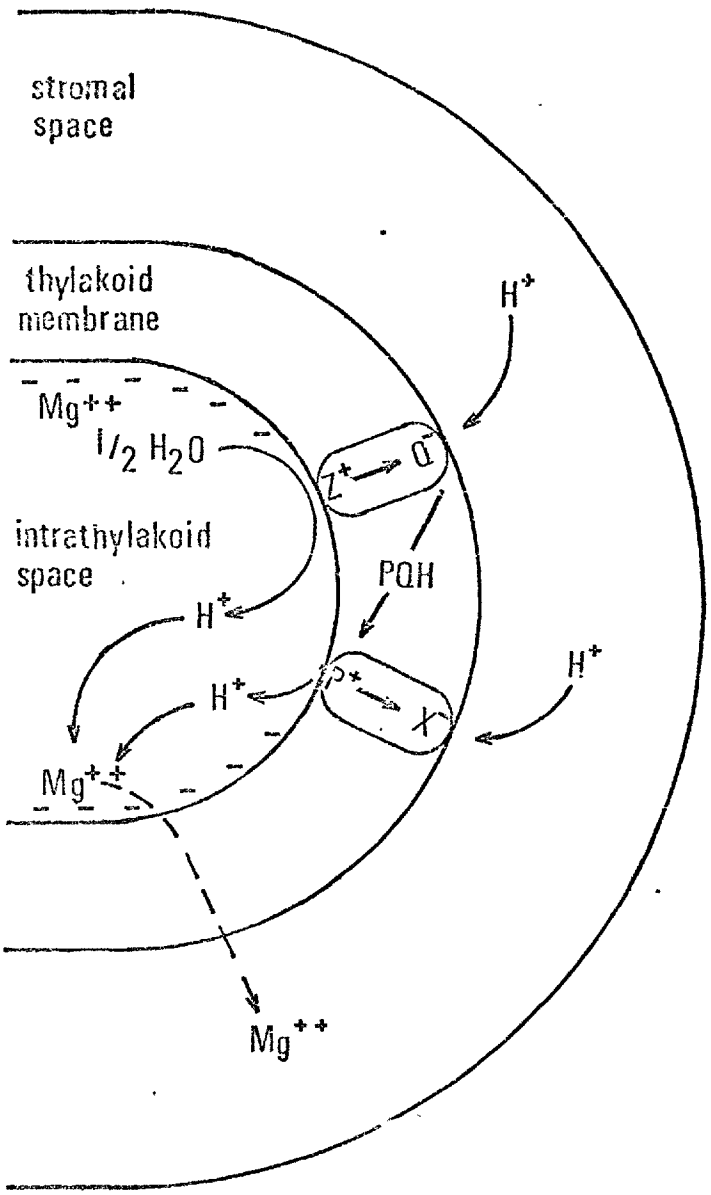


Fig. 1.3 Schematic representation of part of an intact chloroplast showing the vectorial arrangement of electron transport components inthe thylakoid membrane. Illumination is proposed to result in the electrogenic transport of two protons from the stromal to intrathylakoid spaces per electron transported from H_2O to X. This may in turn induce secondary ion transport and efflux of Mg^{2+} from granal to stromal compartments.

Such transmembrane electrical potentials would be expected to decrease the activation energy of the back reaction, if the PSII reaction centres were assymmetrically arranged in the thylakoid as shown in Fig. 1.3. Furthermore, by calibrating the magnitude of DLE increments stimulated by imposed diffusion potentials across the membrane, Barber[75] calculated that the enhancement of DLE observed 1 msec after steady state illumination of chloroplasts had ceased might be partly due to transmembrane electrical potentials of up to 100 mV created in the light.

According to the scheme in Fig. 1.3, the transport of an electron from H_2O to $NADP^+$ subsequent to primary charge separation results in the electrogenic uptake of two protons into the intrathylakoid space.

This proposal has considerable experimental support. Proton uptake from the external medium on illuminating broken chloroplasts was first observed by Jagendorf and Hind[76] and has since been well documented[19,31,46-48,65,67,77] Under steady state illumination conditions, chloroplasts appear to maintain a significant ΔpH gradient of up to three pH units[78,79] and an energetically smaller electrical potential of 50-100 mV.[75,80] This indicates that considerable secondary ion fluxes must occur in response to proton uptake in order to maintain (or approximately maintain) bulk electroneutrality across the thylakoid (see next Section).

Direct evidence has recently been obtained that two proton translocating sites occur in non-cyclic electron transport.[67,81] Furthermore, it has been shown by studies incorporating a rapidly responding pH electrode[82] that protons associated with oxygen evolution are released on the inner side of the thylakoid. These results are consistent with the clear demonstration that two native energy-conserving sites (coupled to ATP synthesis) occur in non-cyclic electron transport, for which evidence has come from the study of partial reactions of the Z-scheme using artificial

electron donors and acceptors.[43,46-48]

Many observations support the idea that a light-induced electrochemical proton gradient is used to support ATP synthesis. Compounds which decrease the overall ability of the thylakoid to maintain ΔpH and $\Delta\psi$ gradients may decrease phosphorylation in parallel. These compounds can act in different ways and are collectively known as uncouplers. Membrane soluble, weak anions, such as FCCP[83] appear to directly transport H^+ out of the thylakoid by protonation of the anion and subsequent diffusion through the membrane of the uncharged acid.

Other uncouplers, known as ionophores,[84-86] increase the permeability of the membrane to ions. Those ionophores of the gramicidin class are extremely effective uncouplers in chloroplasts[31] and appear to form proton conducting channels in the thylakoid, thereby causing the thylakoid to be 'leaky' to protons. Other ionophores appear to increase the cation-permeability of the membrane, and their uncoupling action appears to depend on whether or not they also bind protons.[86] For example, nigericin promotes neutral H^+/K^+ exchange across the membrane and thereby abolishes the ΔpH component of the high energy state (Equation 1.4) without affecting the electrogenic component $\Delta\psi$. In contrast, valinomycin, which increases the membrane permeability to K^+, apparently does not bind protons and therefore cannot by itself promote neutral H^+/K^+ exchange. Under certain conditions when K^+ is initially out of equilibrium, valinomycin may dissipate the electrogenic $\Delta\psi$ by promoting secondary K^+ fluxes without much affecting ΔpH.[86] The relative ability of these latter two ionophores to abolish phosphorylation in photosynthetic membranes should therefore depend on the relative magnitude of ΔpH or $\Delta\psi$ under any given set of experimental conditions. This has indeed been shown to be the case.[31,86,87]

There are two further important observations which support the hypothesis that ATP synthesis utilises the electrochemical energy of a proton gradient.

The striking demonstration by Jagendorf and Uribe[88] that ATP synthesis results from a pH gradient artificially imposed on broken chloroplasts in the dark lends great support to the chemiosmotic hypothesis.

Secondly, there are indications under conditions when ATP synthesis is occurring in illuminated chloroplasts, that a decrease occurs in the steady state extent of the pH gradient.[65,77,89]

From the above observations, there now seems little doubt that chloroplasts can utilise the energy of an electrochemical H^+ gradient to synthesiseATP. Furthermore, the generation of this ion gradient is closely coupled to light-driven electron transport, as clearly seen from the phenomenum of photosynthetic control (the slowing down of electron transport caused by build up of a proton gradient, and its speeding up on utilisation of this ion gradient).[19]

Estimates of the number of ATP molecules phosphorylated for every pair of electrons flowing from H_2O to $NADP^+$ has varied from one to two (see Ref.19 for review). This variation appears to be related to the integrity of the chloroplast preparation, the highest estimates being obtained using broken chloroplasts obtained by disrupting intact organelles immediately before measurements are taken.[19] Since CO_2 fixation requires NADPH and ATP in the ratio 2:3, it has been concluded by some workers[19,80] that enough ATP can be made during non-cyclic electron flow to supply the Calvin cycle without the need for additional operation of cyclic phosphorylation. However, experimental evidence to the contrary exists in the literature[91] and this point is far from settled.

Little is known of the chemistry involved in ATP synthesis even though the ATP synthesising enzyme (or 'coupling factor') has been isolated and purified.[92,93] The coupling factor has been shown to consist of several subunits and shows latent ATP hydrolytic activity. Interestingly, when attached to the membrane and activated to the ATP hydrolysing form, proton

uptake into the thylakoid space occurs which is stoichiometric with the number of ATP molecules split.[93] In many ways, the coupling of proton translocation to ATP'ase activity conforms to the expectations of the chemiosmotic hypothesis operating in the reverse direction.[65] Further work in this area may help in testing some of the predictions of the Mitchell hypothesis.

Although the chemiosmotic scheme seems able to account for most of the features associated with phosphorylation, it is not the only hypothesis concerned with energy coupling in membranes.[61,94-96] Recent work from Good's laboratory[48] suggests that phosphorylation may be intimately associated with the generation of high concentrations of localised pools of protons within the membrane, rather than a general delocalised pH gradient, an idea earlier proposed by Williams.[94] Alternatively, localised charge redistributions on illumination of chloroplasts may be linked to ATP synthesis via local membrane conformational changes,[95] or by conformational changes mediated by cytochromes.[96] Such suggestions may well have to be reconciled with the broader aspects of the chemiosmotic hypothesis as future research probes deeper into the molecular processes involved in ATP synthesis.

1.1.6. Secondary Ion fluxes

The data of Witt indicates that electrogenic proton uptake into the intrathylakoid space associated with a single turnover of the photosynthetic apparatus results in a significant transmembrane potential of 50 mV (inside positive). It is apparant that a few turnovers would soon result in a large membrane potential which would prohibit the entry of further protons and halt electron transport. Since both ΔpH and $\Delta\psi$ can be used to drive phosphorylation,[64] this is of no theoretical concern. However, the chloroplast has been observed to take up large quantities of protons

on illumination, and develop a substantial pH gradient across the membrane[76-78,89] and only a small electrical component in the steady state.[75,80] For this to occur, the membrane potential on continuing proton uptake into the intrathylakoid space must be reduced by secondary ion movement, either in the form of simultaneous anion uptake, or counter-exchange of cations for protons across the thylakoid.

Because of the limiting outer envelope, ion fluxes have generally been measured in broken chloroplasts, and it appears that either anion uptake or cation extrusion from the intrathylakoid space can be observed to balance H^+ uptake depending on what suspending media are used.[65,97-101] In solutions containing high concentrations of completely dissociated small anions such as Cl^-, H^+ uptake of broken chloroplasts on illumination is associated with anion uptake and subsequent swelling of the intrathylakoid space caused by osmotically driven water entry.[98]

Using only non-permeant anions such as galacturonate,[101] solutions of weak anions,[99] or low concentrations of dissociated electrolytes,[100] it seems that cation extrusion from the intrathylakoid space contributes greatly to secondary ion flow. Under these conditions, shrinkage of the thylakoids is observed.

It is clear from the above observations that the composition of the suspending media can determine the nature of secondary ion fluxes across thylakoids. *In vivo*, the physiological media in which thylakoids are bathed is the stroma, which is enclosed by the chloroplast outer membrane. As yet, no method has been devised to directly measure ionic fluxes across *in vivo* thylakoids.

However, isolated intact chloroplasts which retain undamaged outer membranes and show high rates of CO_2 fixation are thought to approximate to the *in vivo* functional state of the organelle.[97] On illuminating such preparations, Heldt and colleagues[102] have estimated from the partition of

a weak acid (DMO) and a weakbase (methylamine), that a pH gradient of 2.5 units was developed across the thylakoids within the intact organelle. Although some proton efflux from the stroma to the suspending medium was observed, it was concluded that the pH gradient mainly reflected an acidification of the intrathylakoid space, and an alkalisation of the stroma brought about by inward proton pumping across the thylakoid membranes. For reasons mentioned earlier, the development of a substantial pH gradient is presumably accompanied by considerable secondary ion flow within the intact chloroplast. The nature of this secondary ion flow will presumably be determined by the ionic content of the stroma which in turn, will be governed by the properties of the outer membrane. Unfortunately, our present knowledge concerning both the ionic levels within the intact chloroplast and the permeability properties of the outer chloroplast membrane is rather unsatisfactory.

Recently, Gimmler et al[11] reported that isolated chloroplasts showing good rates of CO_2 fixation, and estimated to be 70-95% intact by the ferricyanide permeability method,[103] retained high levels of metal cations. They found that K^+, Na^+ and Mg^{2+} in the ratio 2:1:2 respectively were apparently maintained at a stromal concentration greater than that of the suspending medium, indicating a low cation permeability of the outer membrane. Measurement of the internal space of the intact chloroplasts by differential retention of ^3H_2O (permeable) and ^{14}C-sorbitol (impermeable) led to the calculation that the apparant average stromal concentration of these cations was 20-40 mM. Osmotic and radiotracer studies also indicated the impermeability of the outer membrane to these cations, but showed that Cl^- and acetate were able to penetrate more readily.[11] On illumination, no Mg^{2+} or Na^+ fluxes were observed across the outer membrane of intact chloroplasts. However, some K^+ uptake was noted which occurred apparently in response to a small proton efflux

from the intact organelle (also observed by Heldt).[102]

These results[11] conflict with earlier observations made by Nobel[104] who found that preillumination of leaves of Pisum sativum lead to considerable (up to 30%) decrease in both the cationic and Cl^- composition of subsequently isolated chloroplasts. Later however, Lin and Nobel[105] reported that under these conditions, an increase rather than a decrease in Mg^{2+} content of such chloroplasts could be observed. Although considered 'intact',[104] no attempt was made by these workers to characterise the integrity of the chloroplast outer membranes. It is possible therefore that damage to the outer envelope had occurred during chloroplast isolation, and that the permeability properties of the outer membrane had thereby been altered. For this reason it is considered that these results[104,105] are less convincing that those of Gimmler.[11]

Clearly, further work in this area is required before firm conclusions concerning the permeability of the outer chloroplast membrane can be drawn.

Most workers seem to be of the opinion that the stromal concentration of cations can be quite high, in the region of 10-100 mM for each cation.[65,97,104-106] It is possible, therefore, by drawing on the results of experiments with broken chloroplasts, to hypothesise on the nature of light induced ionic fluxes which occur in the intact chloroplast. For several reasons, it seems more likely that the proton uptake into the intrathylakoid space would be accompanied by metal cation efflux from the granal to stromal compartments rather than anion uptake.

Firstly, much of the stromal anion is apparently in the form of negatively charged proteins which would hardly be expected to be permeable to the thylakoid membrane.[11] Secondly, the presence of these proteins appears to result in a significant $Donnan$ potential[11,97] between the stroma and the external medium which would tend to reduce concentrations of small, permeable anions within the intact organelle. Thirdly, extensive anion

(Cl^-) uptake in response to proton pumping in broken chloroplasts is associated with swelling of the intrathylakoid space.[98] However, *in vivo* thylakoids have been observed to shrink on illumination,[104-109] indicating little or no net uptake of solute from the stroma.

Although it seems unlikely that extensive anion transport occurs on illumination of intact chloroplasts, such anion fluxes cannot be ruled out, especially since the outer membrane is permeable to Cl^- and acetate.[11]

Assuming that considerable cation fluxes occur in *in vivo* chloroplasts, and further assuming that the concentrations of monovalent and divalent cations in the stroma are equal, then the data of Hind *et al*[100] suggests that divalent cation movement would occur in preference to monovalent fluxes. It has been estimated that *in vivo* light-driven cation fluxes could increase the stromal concentration of Mg^{2+} by 10-15 mM if this cation predominantly balances proton uptake into the thylakoid space.[97,100,105] (See Fig. 1.3.)

In recent years, there have been a number of observations that varying the ionic composition of the suspending media induces changes in the structure and function of isolated chloroplasts which do not retain their outer membranes. Some of this work will be reviewed in the following sections.

No doubt some of these ion induced effects on broken chloroplasts result purely from the use of non-physiological suspending media. However, others may have considerable physiological significance in view of the possibility of changes in the ionic composition of the stroma brought about by light-induced ion fluxes *in vivo*. It is of some importance, therefore, to determine the exact nature of such light induced ion fluxes. Consideration of these points prompted many of the experiments reported in Section 3 of this thesis.

1.1.7. CO_2 fixation: possible regulation by ion fluxes *in vivo*

The fixation of carbon dioxide involves a complex series of enzyme-mediated biochemical reactions known as the Calvin cycle,[1] and which are located in the stromal space. It was not until the importance of the outer envelope in maintaining stromal integrity was realised, that rates of CO_2 fixation by intact isolated chloroplasts began to match those observed in the living plant.[16-18] The overall reaction can be written thus:

$$n\ CO_2 + 2n\ NADPH + 3n\ ATP \rightarrow (CH_2O)_n + 2n\ NADP^+ + 2n\ H_2O + 3n\ Pi + 3n\ ADP^+ \qquad \text{Eq. 1.5}$$

The primary CO_2 acceptor is Ribulose 1-6 Diphosphate (RuDP), and its carboxylation is catalysed by RuDP carboxylase (E.C. 4.1.1.39). For every three RuDP molecules carboxylated, six triosephosphates are formed. Five of these undergo a complex series of reductive aldol condensations and transketolation reactions to regenerate three RuDP, whilst the sixth triose phosphate represents net product. These reactions constitute the Calvin cycle which is catalytic for CO_2 fixation, and auto-catalytic if the sixth triose is fed back into the cycle.[110]

As in any metabolic pathway a complex series of control mechanisms exist[111] to coordinate the various steps in the cycle and integrate CO_2 fixation with the rest of the chloroplast and cellular metabolism.

One such mechanism may be linked to possible ion fluxes which occur at the onset and termination of illumination. It has been found,[110] that CO_2 fixation ceases rapidly (less than 30 seconds) after light is terminated, even though adequate supplies of CO_2 and its acceptor RuDP are apparently available. Since RuDP carboxylase has been found[110,111] to have a sharp pH activity profile and be allosterically activated by Mg^{2+}, it appears that alkalisation and an increase in Mg^{2+} content of the stroma in response to H^+ uptake into the thylakoids may 'switch on'

this enzyme on illumination of intact chloroplasts and leaves; and that the opposite process on darkening may switch off CO_2 fixation. The advantages of this would be twofold. First, Calvin cycle intermediates would exist in appreciable quantities in the dark, and help prevent serious lags in CO_2 fixation[110] caused by the necessity to synthesise the considerable pool of intermediates before net CO_2 fixation for export from the chloroplasts could begin. Even so, appreciable delays in CO_2 fixation have been observed[110] on first illuminating intact chloroplasts which are considerably reduced by external addition of intermediates of the Calvin cycle.

Secondly, it can be seen that without control, the Calvin cycle can run in the dark on NADPH and ATP indirectly replenished from the cytoplasm (from glycolysis and oxidative phosphorylation) via the translocases in the outer envelope. This would result in a futile cycle between the chloroplast and the mitochondria. In fact, chloroplasts can be manipulated to demonstrate dark CO_2 fixation,[2] but only when the stromal pH is (indirectly) raised to pH8. A stromal pH of 7.3 completely inhibits this, suggesting that control through pH-sensitive enzymes (RuDP carboxylase and some phosphatases catalysing intermediate steps[112]) is exerted by light/dark proton fluxes.

The occurrence of ion fluxes in the intact chloroplast is investigated in Section 3 in view of the proposed mechanisms controlling CO_2 fixation.

1.2. Chlorophyll a fluorescence

1.2.1. Chlorophyll a fluorescence: an intrinsic probe of photosynthesis

Under optimal conditions, most of the light absorbed by photosynthetic organisms is used to drive the primary photochemical reactions outlined in earlier sections. However, a smaller part of the absorbed excitation energy is inevitably not used for this purpose, and is dissipated as heat or re-emitted as chlorophyll a fluorescence. *In vitro* chlorophyll mediated photochemical reactions in organic solvents are thought to involve mainly excited triplet states of the pigment.[113,114] The failure to observe chlorophyll triplet states of the pigment in the chloroplast (see, for instance, Ref.115), has generally led to the assumption that an electron is transferred to the primary acceptor from the singlet excited state of the reaction centre chlorophyll.[116]

Since emission as fluorescence represents an alternative pathway of chlorophyll singlet de-excitation, measurement of the relative yield of fluorescence has been an extremely useful intrinsic probe of the primary photochemical reactions of photosynthesis. In addition, both yield and other measurable parameters of fluorescence (excitation and emission spectra, polarisation, etc.) depend both on the physical characteristics of the immediate environment, and on the mutual distance and orientation between fluorescing molecules. Chlorophyll fluorescence measurements, therefore, may also yield information on the environment of the pigments in the thylakoid, and hence any structural changes which occur in the membrane.

1.2.2. Origin of chlorophyll a fluorescence

Careful analysis of low temperature ($77^{\circ}K$) absorption and fluorescence spectra of chloroplasts has revealed the presence of chlorophyll a in

several different forms in the thylakoid membrane[20,114,117-120] These forms are characterised by slightly different wavelengths of the absorption maxima in the red region of the spectrum, and presumably reflect different associations of chlorophyll a with proteins (and lipids) in the membrane (see Section 1.1.4). At room temperature, fluorescence originates mainly from the chlorophyll associated with PSII.[119-122] The emission spectrum shows a single broad band centred around 685 nm together with a shoulder at 710-740 nm. The chlorophyll a species mainly responsible for this emission is probably that form which has an absorption maximum at 678 nm (Chl_{678}). Other light harvesting pigments of PSII (such as chlorophyll b and Chl_{670}) do not fluoresce, but appear to efficiently transfer absorbed excitation energy to Chl_{678}[117,118] An exception to this is to be found in the red and blue-green algae, where weak fluorescence of the phycobilins is also detected in the room temperature emission spectra.[117]

The light harvesting pigments of photosystem I appear to efficiently transfer absorbed excitation energy to a form of chlorophyll a which is only weakly fluorescent at room temperature.[120,123] Analysis of emission and excitation spectra[120-124] has indicated that fluorescence from pigments of PSI may make some small contribution to the total emission spectrum at room temperature, especially in the 710-740 nm region.

On lowering the temperature to that of liquid nitrogen (77°K) and below, the fluorescence emission spectra becomes more complex both for isolated chloroplasts and intact organisms.[117,118,120-122,125-128] Two peaks associated with PSII can be discerned at 685 nm (F685) and 695 (F695). In addition, another chlorophyll a species (or probably more than one[117,118]) becomes strongly fluorescent at these temperatures, with a broad emission band centred around 730 nm (F1). The exact assignment of these fluorescence bands to various chlorophyll a forms in the membrane

is not yet fully clear. Based to some extent on observations that F695 only appears when PSII reaction centres are closed,[126] and is particularly sensitive to inhibitors of non-cyclic electron transport,[127] it has been proposed that F695 originates as emission from PSII reaction centre chlorophyll a and F685 from the associated light harvesting chlorophylls of PSII (see also Refs.117 & 118). More recently, Butler and Kitajima[128] have suggested that F685 originates from the chlorophylla/b light harvesting pigment protein complex recently isolated by Thornber (Ref.58, and see Section 1.1.4). This complex is thought to transfer absorbed excitation energy mainly to the antennae chlorophyll a of the PSII reaction centre. According to Butler and Kitajima, F695 is emitted by the light harvesting chlorophyll a directly associated with PSII traps, and F1 represents emission exclusively from PSI light harvesting chlorophyll a.

This proposition gains broad support from characterisation of the excitation and emission spectra of sub- chloroplast fractions thought to represent PSII, PSI and chlorophyll a/b pigment/protein complexes.[58-60,128-130] However, Mohanty et al[131] after allowing for self absorption of fluorescence, found that the P700-chlorophyll a protein derived from PSI fluoresced at 685 nm as well as 730 nm. It must be borne in mind, however, that complete purification of this complex may not yet have been achieved, and that the emission detected at 685 nm may be due to contamination of highly fluorescent chlorophyll a of PSII. Also it is by no means certain that the emission spectra of these isolated chlorophyll/protein complexes faithfully represent the 'true' spectra in the thylakoid membrane. Structural modification of these complexes may well have occurred during isolation which could subtily alter the emission characteristics of the associated chlorophyll.

Whilst bearing these problems in mind, it is reasonable to conclude that the bulk of experimental evidence[120-122] indicates that the room

temperature fluorescence yield of chloroplasts provides a monitor of excitation energy levels in PSII. With reservations, low temperature spectra may yield information of the transient levels of excited pigments in PSII (F685, F695) and also of PSI (F1).

1.2.3. Fluorescence yield

The relative yield of chlorophyll a fluorescence has been extremely useful in kinetic studies of the primary phctochemical reactions over many orders of magnitude of time. Since fluorescence results from de-excitation from the singlet excited state, the relative yield of this emission will be dependent on the magnitude of all other competing pathways for singlet de-excitation. Considering for the moment only the fluorescent chlorophyll a of PSII (at room temperature) the fluorescence yield (ϕ_f) can be described by equation 1.6 (see Ref.116):

$$\phi_f = \frac{I_f}{I} = \alpha \left(\frac{K_f}{K_f + K_h + K_t + K_p f(Q)} \right) \qquad \text{Eq. 1.6}$$

where I_f is the intensity of fluorescence emission, and I the total intensity of absorbed light; α is the fraction of light absorbed or transferred to the fluorescing chlorophyll of PSII. K_f, K_h and K_t are first order rate constants assumed to describe de-excitation by fluorescence, radiationless de-excitation (other than trapping) and transfer of excitation energy to PSI respectively. K_p is the first order rate constant for trapping of the exiton by a single open reaction centre. It is assumed in addition that only open reaction centres can accept excitation energy for trapping. The rate of de-excitation of singlet excitation energy by photochemical trapping will be some function of the relative proportion of open traps f(Q) (discussed in more detail in Section 1.2.4.). It is clear from Equation 1.6 that at constant illumination intensity

a change in either of the rate constants K_t or K_h or of K_p f(Q) or α results in changes of fluorescence yield.

In fact illumination at high incident intensity of dark adapted photosynthetic organisms results in a complex series of fluorescence yield changes over a period of minutes as shown in Fig. 1.4. Known as fluorescence induction, or the 'Kautsky effect'[132] these fluorescence changes occur in a characteristic way (labelled OIDPSMT after the nomenclature of Lavorel[133] and Munday and Govindgee[134]). The rate and magnitude of the fluorescence transients which occur during induction can vary somewhat between different photosynthetic organisms.[132-135] It has generally been possible, however, to divide these transients into fast changes (mainly O → P) which occur within seconds and mainly reflect changes in the efficiency of trapping by PSII, and slow changes (P → T) which do not seem to be dependent on the rate of trapping but may reflect changes in α, K_t K_p or K_h.

1.2.4. Dependence of fluorescence yield on the rate of trapping at PSII

The important work of Duysens and Sweers in 1963[136] provided the basis of present understanding of the relationship between fluorescence yield and the yield of photochemical trapping at the PSII reaction centres. They found that illumination of algae with light preferentially absorbed by pigments of PSII (PSII light) resulted in an increase in chlorophyll *a* fluorescence. On superimposing PSI light, however, the intensity of this emission was decreased. In the presence of DCMU, which inhibits non-cyclic electron flow just after the primary acceptor of PSII, PSII light induced a larger increase in fluorescence which was not reversed on adding a second beam of PSI light. They explained their results by considering the antagonistic reduction and oxidation of the PSII reaction centre which they termed 'Q'. In the oxidised form, 'Q' was able to accept excitation energy from the bulk chlorophyll of PSII, and therefore acted as an

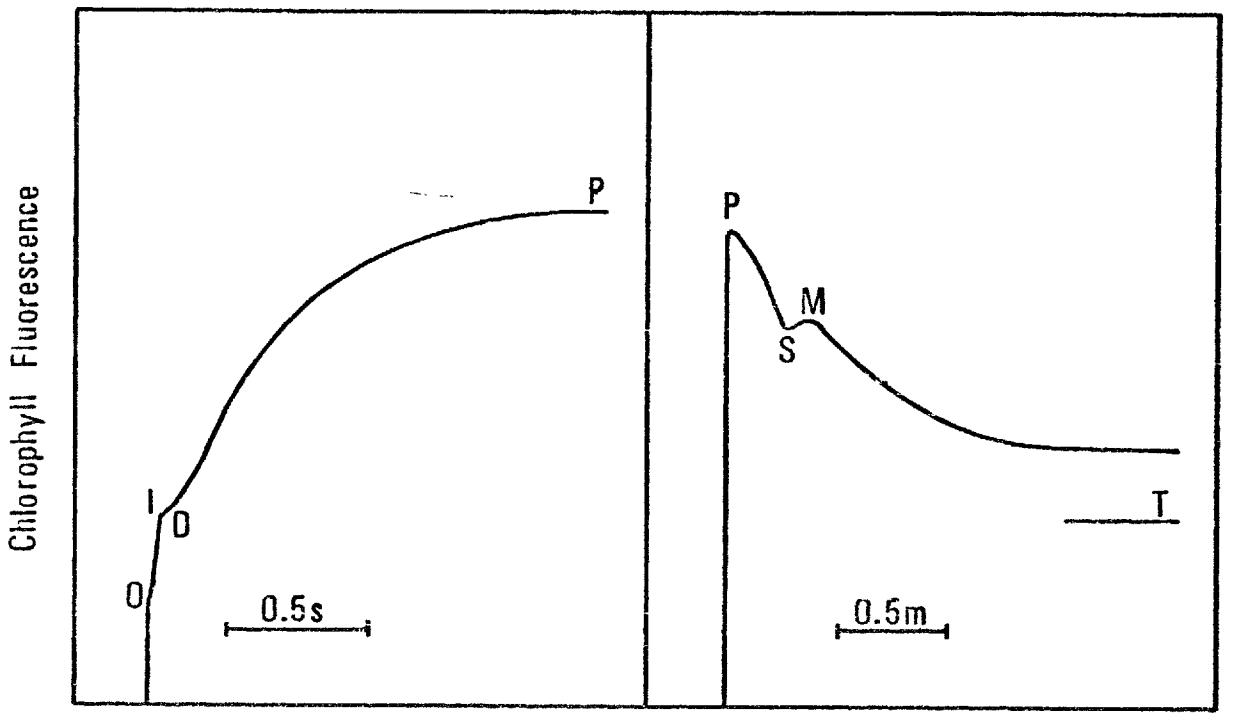


Fig. 1.4 Chlorophyll fluorescence transients observed on illuminating a dark pretreated spinach leaf with high intensity (blue/green) light(20 Wm^{-2}). Note the relatively fast(OIDP) and slow (PSMT) components.

as an efficient quencher of PSII fluorescence. Overall reduction of 'Q' in PSII light closed the reaction centres. In the closed form, 'QH' was unable to accept further excitation energy and fluorescence from PSII was consequently high. Reoxidation of 'QH' by PSI light (blocked by DCMU) re-opened the PSII traps and quenching of fluorescence again occurred.

In present day terms the 'Q' of Duysens and Sweers[136] is taken to be, not the reaction centre complex *in toto*, but the primary electron acceptor, Q. The same explanation, however, is still in use. In the oxidised form, Q reflects the fact that the reaction centre is open and can accept exitons (low fluorescence), whilst when reduced to Q^-, the trap is closed until secondary electron flow reoxidises the primary electron acceptor.

As discussed in Section 1.1.4., secondary electron flow in PSII occurs in the following sequence:

$$Y(Chla^{*}_{II}\ Q)A \rightarrow Y(Chla^{+}_{II}\ Q^{-})A \rightarrow Y^{+}(Chla_{II}\ Q^{-})A \rightarrow Y^{+}(Chla_{II}\ Q)A$$

state of trap	open?	closed	closed	open
expected fluorescence yield	low?	high	high	low

Eq. 1.2.

Because reoxidation of Q^- is generally much slower than reduction of $Chla^{+}_{II}$,[31] reopening of the PSII trap is normally determined by the kinetics of secondary electron flow on the reducing side of PSII.

The Q hypothesis of Duysens and Sweers has been reasonably successful in describing the fast fluorescence induction O → P which occurs within the first few seconds of illuminating dark adapted organisms. This has been termed variable fluorescence yield.

However, the initial rise to the O level apparently shows little dependence on primary photochemical reactions of photosynthesis and has been termed the 'constant' yield of fluorescence (or F_o). This invariable

fluorescence probably originates from three sources. Firstly, Lavorel[124] found a prominent 720 nm component in the excitation spectra of the O-level fluorescence yield suggesting that this emission may include a contribution from the chlorophyll associated with PSI. Secondly, it has been suggested on theoretical grounds that part of F_o may reflect the fact that even when all of the PSII traps are open, trapping of absorbed light energy is not 100% efficient and so some de-exitation occurs via fluorescence.[39,137,138] Thirdly, F_o may include fluorescence from chlorophyll *a* totally unconnected with any active reaction centres. It is not clear (and is probably variable) to what extent these three source contribute to F_o.

The subsequent rise from O → P during fluorescence induction *in vivo* appears to reflect the overall reduction of intermediates in the electron transport chain which eventually results in reduction of Q and closing of PSII traps.[121,122] This occurs because CO_2 fixation does not commence immediately on illumination (see Section 1.1.7). The ultimate pool of electron acceptor ($NADP^+$) therefore becomes reduced, limiting the ability of PSI to 'keep pace' with PSII. During the O → P phase, a transient O_2 evolution occurs (O_2 'gush'), the quantum yield of which is approximately inversely proportional to that of fluorescence.[121] This important observation led Lavorel to state (e.g. see Ref.137) that competition between fluorescence and trapping for excitation energy within PSII was the 'fundamental alternative'. In mathematical terms, this is given by Equation 1.7:

$$\frac{\phi_f}{\beta} + \phi_p = 1 \qquad \text{Eq. 1.7}$$

where ϕ_p is the quantum yield of trapping averaged throughout PSII and β is a constant. This rationale forms the basis of any quantitative analysis of the relationship between the yields of photochemical trapping and fluorescence (see Section 1.2.6.3).

The O → P phase of fluorescence induction is also clearly observed in isolated chloroplasts not retaining their outer envelopes and in the absence of added electron acceptors. Various results indicate that the variable yield of fluorescence in such broken chloroplasts is also dependent on the oxido-reduction state of Q. In the absence of electron acceptors, the rate of O → P transient is increased with increasing light intensity, or by addition of DCMU, both of which prevent net reoxidation of $\bar{Q}$.[139] The presence of electron acceptors such as ferricyanide[139] which maintain Q in the oxidised form also abolish the O → P increase, whereas chemical reduction of Q with dithionite[140] maintains fluorescence at a high yield.

Overall, therefore, it is reasonably well established that the variable yield of chlorophyll fluorescence can be controlled by the proportion of open to closed PSII reaction centres when reoxidation of Q by secondary electron flow is the factor controlling the re-opening of PSII traps. However, under certain conditions, the open and closed states of the traps can be determined by events on the oxidising side of the reaction centre. This occurs when the endogenous electron donor to the reaction centre chlorophyll $\underline{a}_{II}$ is inactivated by treatments such as washing of chloroplasts in high concentrations of tris buffer,[141] ultraviolet irradiation,[142] or mild heating of the membranes to 50^{o}.[143] Such treatments inhibit oxygen evolution and presumably considerably retard re-reduction of the oxidised chlorophyll $\underline{a}_{II}$ trap. Under these conditions the variable fluorescence yield is observed to be low, even though the PSII traps would be expected to be closed.[142,143] At first sight, this observation appears to contradict the hypothesis of the 'fundamental alternative' between fluorescence and photochemical yields, since in such inhibited chloroplasts, both of these quantities are reduced.

A possible explanation for this discrepancy may lie in the ability of chlorophyll $\underline{a}_{II}$, even in the oxidised form, to accept excitation energy from

the light harvesting pigments of PSII. This excitation would then be degraded thermally. If the pigment trap chlorophyll $\underline{a}_{II}$ exists in the form of a dimer, as suggested by EPR spectroscopy[30] it is not difficult to imagine that photooxidation of one chlorophyll molecule in the dimer may disturb the excitation energy levels in the complex so that subsequently, excitons delivered to the trap (in the photooxidised form) are efficiently degraded to heat by internal conversion. Under normal conditions, when the trap is closed (but $Chl\underline{a}_{II}$ is reduced) excitation energy delivered to the reaction centre may be efficiently transferred back to the light harvesting chlorophyll of PSII, from which it has a high probability of fluorescence. This speculative explanation for the low quantum yield in tris-washed chloroplasts is supported by the observation that on addition of artificial electron donors to PSII, the high variable fluorescence yield changes are again restored. Such electron donors would be expected to maintain chlorophyll $\underline{a}_{II}$ in the reaction centre in the reduced state.[144]

The influence of the oxidising side of PSII on chlorophyll fluorescence yield is also apparent at low temperatures (77^{o}-180^{o}K), where most thermal reactions are inhibited.[145-149] Primary photochemical reactions of PSII may still occur, however. At liquid nitrogen temperatures ($\sim 77^{o}$K), the following events are thought to occur at the PSII reaction centre:[148]

$$\text{Cyt b559. } Chl\underline{a}^{*}_{II}\text{. } Q \rightarrow \text{Cyt b559. } Chl\underline{a}^{+}_{II}\text{. } Q^{-} \rightarrow \text{Cyt b559}^{+}\text{. } Chl\underline{a}_{II}\text{. } Q^{-} \qquad \text{Eq. 1.8}$$

At these temperatures (but not at room temperature), Cyt b559 replaces components of the water splitting enzyme as electron donor to chlorophyll $\underline{a}_{II}$. Q reduction can be measured by an absorbance change at 550 nm whilst oxidation of Cyt b559 also results in an absorption change at 559 nm.[145-149] Extensive kinetic studies of this low temperature PSII reaction performed by Butler and co-workers[146-148] have clearly shown that the fluorescence induction on first illuminating dark, frozen samples of chloroplasts, cor-

relates with the photoxidation of Cyt b559, not reduction of Q. This again suggests that the high fluorescence yield associated with closed reaction centres of PSII is only evident on the reduction of $Chl\underline{a}^{+}_{II}$.

The possible role of chlorophyll $\underline{a}^{+}_{II}$ as an efficient quencher of chlorophyll a fluorescence in PSII has been stressed here for one important reason. Although fluorescence yield of PSII can be correlated with the quantum yield of trapping at the reaction centres under many conditions, no such correlation has been shown for PSI. Vredenberg and Slooten[149] have shown that PSI fluorescence is insensitive to the rate of electron flow through the PSI reaction centres. However, because of the high negative redox potential of the primary electron acceptor X,[150] secondary reoxidation occurs extremely rapidly at room temperatures,[151] and the reopening of PSI traps is always controlled by the reduction of $Chl\underline{a}^{+}_{I}$. By analogy with photosystem II, therefore, if $Chl\underline{a}^{+}_{I}$ is an efficient quencher of chlorophyll fluorescence then it would be expected that PSI fluorescence yield would be insensitive to the redox state of the PSI reaction centres. On the basis of the above reasoning it would be expected that a closed reaction centre of PSI in the form $(Chl\underline{a}_{I}X^{-})$ would be highly fluorescent. To my knowledge, however, no fluorescence measurements have yet been performed where it has been established that the PSI traps are in this theoretically non-quenching form.

Bearing in mind the possible influence of the oxidising side of PSII under certain conditions, the idea that the redox state of Q is the main factor controlling both fluorescence yield and the rate of photochemical trapping in PSII has been of great use in fluorescence studies designed to investigate the primary photochemical reactions of photosynthesis. However, the slower fluorescence yield transients which occur during induction ($P \rightarrow T$, see Fig. 1.4) appear to be less dependent on the quantum yield of photochemical trapping, and other explanations for these phenomena have to

be sought.

1.2.5. Slow fluorescence induction *in vivo*

It has been shown by Bannister and Rice[152] that the slow PSMT phase of fluorescence induction is associated *in vivo* with a steady increase in the rate of O_2 evolution which reaches a maximum at the point T. Qualitatively, therefore, the overall decline in fluorescence yield reflects an overall increase in the photochemical utilisation of available excitation energy as CO_2 fixation becomes fully efficient. However, closer examination of these phenomena reveals that the competition between fluorescence and trapping for excitation energy is not the only cause of those slow induction phenomena.

The P → S fluorescence decline is abolished by anaerobis and decreased temperature,[135] and is generally more sensitive to physiological stress applied to the organism than is the Q-dependent OIDP phase.[122] However, since these treatments would also be expected to inhibit CO_2 fixation, these results may not be as significant as previously thought.[121,122,135] More important is the observation that in the presence of DCMU and high light intensities (which result in permanent closure of the PSII traps), similar slow fluorescence yield changes can still be observed.[152-154] Under these conditions, the fluorescence yield changes cannot be controlled by fluctuations in the rate of photochemical trapping by PSII. However, addition of uncouplers which penetrate the *in vivo* organism abolishes the slow fluorescence induction.[122] This seems to indicate that P → T yield changes are somehow linked to events associated with phosphorylation which in the presence of DCMU can still be supported by cyclic electron flow around PSI.[152-154]

To explain these induction phenomena, Bonnaventure and Myers[155] and Murata[156] independently produced models which considered the involvement of

changes in other dissipative pathways available to excitation energy (α, K_t, K_h). In a penetrating study of simultaneously measured O_2 evolution and fluorescence yield in Chlorella, Bonnaventura and Myers concluded that the M → T phase represented a redistribution of light energy between the two photosystems.[155] These authors used a modulated monochromatic light beam at a wavelength preferentially absorbed by PSII to excite fluorescence and induce oxygen evolution, both of which were modulated in phase with the PSII light. The quantum yield of the PSII light could be measured therefore even in the presence of a second light beam, providing that this second beam was not modulated. By selecting a wavelength of light preferentially absorbed by PSI for the unmodulated light (PSI light), they were able to directly measure the enhancement effects of the PSI beam on the quantum yield of the modulated PSII beam. Enhancement was detected as a rapid increase in modulated oxygen evolution and a rapid decrease in modulated fluorescence yield brought about by increased utilisation of the excess quanta delivered to PSII by the PSII beam when PSI light was also given to the algae (a consequence of the push-pull nature of the Z-scheme).

Using this technique, they found that the M → T induction occurred only in the presence of continuous (modulated) PSII light. At the onset of this phase (point M), enhancement was high, but as fluorescence declined towards T, enhancement effects became less marked, and an overall increase in oxygen evolution (in the absence of PSI light) occurred. They called the high fluorescing (high enhancement) condition State I, and the low fluorescing (low enhancement), State II. Their results indicated that continuous excitation of algae by PSII light results in an adaptive change, the State I to State II transition (signalled by M → T fluorescence decline) whereby some of the excess quanta initially absorbed and retained by PSII in State I are diverted to PSI in State II. This has the effect of reducing the high initial imbalance of excitation energy between the two photosystems and

thereby increasing the efficiency of electron transport.

Furthermore, they found that if PSI light was left on for 4-5 minutes at point T, the reverse sequence of events occurred and the algae were restored to the high fluorescing condition (point M). They thus concluded that light absorbed preferentially by PSII drives the State I → State II transition, whilst PSI light drives the State II → State I sequence.

The State I/II changes appears to be an *in vivo* adaptive mechanism whereby electron flow may be optimised by appropriate redistribution of light between the two photosystems to suit any particular illumination condition. Such a mechanism would be of particular use in both land and water based photosynthetic organisms where shading may alter the spectral quality of light reaching the plant or algae.

Murata, using the algae *Porphyridium*, also correlated the M → T induction phase to a redistribution of excitation energy between PSI and PSII.[156] He found that the decrease in room temperature fluorescence correlated with a similar decrease in PSII fluorescence at low temperatures ($77^{o}K$) together with an increase in the emission of PSI at $77^{o}K$. Murata concluded that an increase in K_t, the transfer of excitons from the light harvesting pigments of PSII to those of PSI, was responsible for the increased quantal efficiency of PSI at the apparent expense of PSII. Bonnaventura and Myers did not ascribe the fluorescence changes to any particular mechanism, but suggested that an alteration of only 10% in the fractional absorbance of incident light by PSII (i.e. α) would be sufficient to account for the State I/II hypothesis.

Both changes in α and K_t can presumably be brought about by changes in the structural arrangement of the pigments in the thylakoid membrane. The transfer of excitation energy between chlorophyll *a* molecules by a Förster-resonance induction mechanism depends both on the distance and orientation between donor and acceptor molecules, as well as the degree of

overlap between the emission and absorption spectra.[29]

An increase in K_t (spillover model), might occur if PSI and PSII were physically moved closer to each other by conformational changes within the membrane.[157] Alternatively, a change in α may be brought about by a change in the distance or orientation of a group of light harvesting molecules so that excitation energy could be transferred either to PSII (State I) or PSI (State II).

The concept that emerges from these studies is that a photosynthetic unit of PSII and PSI is not a static entity, but should be thought of as a variable structure, dependent on the physical state of the membrane. Clues to the way in which adaptive State I/II transitions may involve changes in α or K_t, linked to the structural changes in the thylakoid membrane have come from studies of slow, Q-independent fluorescence changes in isolated broken chloroplasts.

1.2.6. Slow fluorescence changes in broken chloroplasts

When isolated chloroplasts not retaining their outer envelopes are treated with DCMU in the dark and then illuminated, a rapid OIP fluorescence increase is observed which can be correlated with the closing of PSII reaction centres.[139] Under these conditions and using high enough light intensities, Q remains reduced (and the traps closed) and such chloroplasts show little subsequent slow fluorescence yield changes. However, certain treatments to such DCMU-poisoned systems can restore Q-independent variations in the yield of fluorescence which bear marked resemblances to certain features of slow induction phenomena *in vivo*. Clearly, these reconstituted fluorescence changes cannot be due to changes in the trapping efficiency of PSII, but do point to the ways in which membrane structural changes may alter other rate constants for chlorophyll *a* singlet de-excitation.

1.2.6.1. Effect of high energy state on chlorophyll a fluorescence in broken chloroplasts

Murata and Sugahara[158] first showed that the cofactor PMS, which stimulates coupled cyclic electron flow around PSI in illuminated DCMU-treated broken chloroplasts, also induces a decrease in chlorophyll a fluorescence yield at physiological temperatures. This decline in fluorescence in the light was reversed on darkening the chloroplasts, or by adding uncouplers such as methylamine and FCCP which also abolish the transthylakoid electrochemical proton gradient.

Using the cofactor DAD in place of PMS, Wraight and Crofts[159] clearly showed by the use of ionophores which selectively abolish the ΔpH or $\Delta\psi$ component of the high energy state (HES) that fluorescence quenching occurred in response to net proton uptake into the intrathylakoid space. The establishment of the pH gradient as the cause of chlorophyll fluorescence lowering was confirmed by Cohen and Sherman who used a variety of methods to reduce net H^+ uptake of chloroplasts and found that fluorescence quenching was inhibited in parallel.[160]

The HES induced fluorescence quenching in broken chloroplasts shows similarities to slow fluorescence induction in vivo, both in regard to the sensitivity to uncouplers, and that coupled cyclic electron flow can support both kinds of fluorescence change. However, analysis of the low temperature emission spectra indicates that PMS stimulated fluorescence quenching decreases the yield of both PSII and PSI fluorescence.[158] In contrast, State I to State II transitions increase low temperature PSI quantal efficiency at the expense of PSII.[156] It seems possible, therefore, that HES fluorescence quenching induces some conformational change in the membrane which increases the non-radiative dissipation (K_h) of excitation energy of both PSI and PSII. The involvement of membrane structural changes in this process has been demonstrated by Mohanty et al.[161] They found that fixation of chloroplasts

in glutaraldehyde prevents conformational changes of the thylakoid and inhibits PMS induced fluorescence lowering, even though these membranes retain (to about 40%) the ability to take up protons from the medium.

Recently, work by Papageorgiou and colleagues[122,162,163] has provided some insight into the way the transmembrane pH gradient may influence chlorophyll fluorescence both in vivo and in broken chloroplasts. They found that oxygen was necessary to observe the full extent of PMS stimulated fluorescence quenching. On making a sample of DCMU treated chloroplasts anaerobic, PMS-catalysed fluorescence lowering on illumination was only half that seen in air.[162] Furthermore the full extent of the fluorescence yield changes could be restored simply by re-equilibrating the anaerobic sample with air. They concluded that protonation of the thylakoid membrane induced ultra-structural membrane conformational changes which enhance the diffusion of non-photochemical quenchers of electronic excitation energy (such as molecular oxygen) to the chlorophyll bed. Although this process cannot account for State I/II changes associated (mainly) with M → T phase, a similar mechanism may be involved in the P → S decline in fluorescence which is concomitant with increased oxygen evolution in vivo.

1.2.6.2. The effect of cations on chlorophyll a fluorescence yield in broken chloroplasts

A large number of observations have accumulated in recent years which suggest that the ionic composition of the suspending media of broken chloroplasts can induce changes in the primary photochemical reactions of photosynthesis. Many of these effects appear to be due to the specific interaction of a cation or anion at defined sites. For example, Mn^{2+},[37] Cl^{-}[164] and HCO_3^{-}[165] are required for efficient operation of the water splitting apparatus of PSII, whilst Cd^{2+} inhibits PSII on the oxidising side[166] and CN^{-} inhibits electron transport at the site of plastocyanin reoxidation.[167]

However, cations in general appear to induce a whole series of effects

on the primary reactions of photosynthesis in broken chloroplasts which bear remarkable resemblances to those which occur during State I/II changes in vivo. In this and the next few sections, these effects of cations on fluorescence will be described, and pertinent results from other work briefly discussed in Section 1.3. The physical mechanism by which cations interact with the thylakoid membrane has been the object of extensive study in this thesis, and relevant literature regarding cation-induced structural changes in the thylakoid will be reviewed in Section 4.1.

The influence of cations on the yield of chlorophyll a fluorescence from DCMU treated isolated, broken chloroplasts was first noted by Homann.[168] Under conditions of low ionic strength, it was shown that $MgCl_2$ greatly increased the yield of chlorophyll fluorescence in a manner which could not be ascribed to changes in the yield of photochemical trapping. Independently, Murata and co-workers[157,169,170] confirmed and extended these observations. They found that the cation induced increase in fluorescence was relatively unspecific, with several divalent (Mg^{2+}, Ca^{2+}, Mn^{2+}) and monovalent (K^+, Na^+ methylamine) being effective. However, as noted by Homann,[168] divalent cations induced the maximum increase in fluorescence at much lower concentrations (2 mM) than monovalent cations(100 mM) whereas little or no dependency on the nature or valency of the anion could be detected. Murata also investigated the low temperature spectra of broken chloroplasts in the high and low fluorescing states.[157] He found that an increase in room temperature fluorescence on addition of cations correlated with an increase in the emission of PSII and a decrease in that of PSI at $77^{\circ}K$. It was concluded that low levels of divalent, or higher concentrations of monovalent cations induced a conformational change in the thylakoid which inhibited the spillover of excitation energy from PSII to PSI. Others[161,171] have found that glutaraldehyde fixation of isolated chloroplasts prevents membrane conformational changes and predictably abolishes cation

sensitive fluorescence changes.

The changes in fluorescence yield induced by cations at both room and liquid nitrogen temperatures are similar to those which occur during State II → State I transitions *in vivo*. The implication that cations may play a role in the quantal distribution of light energy to the two photosystems *in vivo* will be discussed in Section 1.5.

More recently, Gross and Hess[172] (confirmed by others[173]) have found that cation sensitive changes in fluorescence yield are more complex than originally thought. These workers took care to wash and resuspend their chloroplast prepations in sugar media containing only sufficient tris-base to titrate the final suspension to pH 8. Under these conditions, the steady state fluorescence emission from DCMU treated chloroplasts was relatively high, and was decreased to a low level on adding monovalent cations (but not divalent cations) up to a concentration of 10 mM. This decrease in room temperature fluorescence correlated with an increase in the F_{730}/F_{685} emission ratio at 77°K indicating that monovalent cations promote spillover of excitation energy from PSII to PSI. Further addition of monovalent cations (100 mM) or divalent cations (3.3 mM) restored fluorescence to the high yield and inhibited spillover as judged from 77°K emission spectra.[172]

The failure of earlier workers[157,168-171] to observe the monovalent cation dependent decrease in fluorescence at very low ionic strength is undoubtedly due to the presence of monovalent cations in the initial suspending media at concentrations which decrease fluorescence to the minimum level.

In view of the high stromal activity of cations[11,97,104-106] it seems doubtful if these fluorescence changes at very low ionic strength have any physiological significance *in vivo*. This point will be discussed further in Section 1.5.

1.2.6.3. Effect of cations on fast fluorescence induction in broken chloroplasts.

Apart from influencing the distribution of excitation energy between PSII and PSI, the cationic content of the suspending media also affects the fast, Q-dependent fluorescence induction which occurs on first illuminating DCMU treated broken chloroplasts. This has been taken as evidence that cations may also influence the transfer of excitation energy within photosystem II, itself. In order to amplify this point, consideration must be paid to the information which can be extracted from analysis of the O to P fluorescence induction in broken chloroplasts.

In the presence of DCMU, this rapid increase on first illuminating thylakoids is assumed to be due to the overall closing of PSII traps by reduction of Q. This is further assumed to occur by a one quantum reaction at each reaction centre. Assuming also the 'fundamental alternative' relationship between trapping and fluorescence, the total area above the curve traced out by O to P is a measure of the number of quanta trapped by the reaction centres (i.e. not emitted as fluorescence), which in turn is related to the total pool of Q reduced during the induction period.[139] This area should not include the constant yield of fluorescence (F_o or O), since this is presumed not to reflect reduction of Q. Briantais et al,[174] Murata[157] and Hipkins[175] found that cations only affect the variable yield of fluorescence. In consequence, the cation stimulated increase in fluorescence yield is also accompanied by an increase in the area above the fast O to P induction curve.[157] However, after normalisation by dividing the area by the variable fluorescence yield, theoretically justified by Hipkins,[175] little difference between the normalised areas in the presence and absence of divalent cations is apparent.[157] This indicates that the pool size of Q has not been changed, nor do cations reversibly activate 'dormant' PSII units.

The shape of the induction curve in DCMU treated chloroplasts also yields information on the relationship of the PSII photosynthetic units to each other. Consider the two following extreme models of the photosynthetic unit (PSU):[137,138]

1. Each PSU consists of a single reaction centre and associated light harharvesting pigments and there is no possibility of a quantum of light absorbed by one PSU being transferred to a neighbouring PSII PSU. In this case light absorbed by a PSU with a closed reaction centre (closed PSU) will have a high probability of re-emission as fluorescence, whilst a quantum absorbed by a PSU with an open reaction centre will have a low probability of fluorescence and a high probability of being trapped. Fluorescence yield will be a linear function of the proportion of closed to open units in PSII. Furthermore, the induction of fluorescence would be purely exponential, dependent on the rate at which the PSII traps are converted from the overall open to closed states. This model is known as the isolated, monocentral or 'isolated puddles' model.
2. In contrast to the isolated monocentral model, the statistical or 'lake' model proposes that there are no morphologically distinguishable photosynthetic units. PSII reaction centres are distributed uniformly throughout the bed of light harvesting pigments at a ratio of one trap per 300 chlorophyll a molecules (see Section 1.1.4). The relationship between fluorescence yield (ϕ_f) and the proportion of open reaction centres (Q) is non-linear and is given by a Stern-Volmer type of expression:[137,138]

$$\phi_f = \frac{I_f}{I} = \alpha\left(\frac{K_f}{K_f + K_h + K_t + K_p\ (Q)}\right) \qquad \text{Eq. 1.9.}$$

where α K_t, K_h, K_p I_f and I have the same meaning as defined in equation 1.6.

The exact dependence of ϕ_f on the quantum efficiency of trapping

depends on the proportion of open traps (Q), the value of K_p, and the rate at which excitons can migrate through the bed of light harvesting pigments. For example, consider the case where migration of excitons is sufficiently fast to enable each exciton to visit every trap in the pigment bed at least once during its lifetime. If, in this case, trapping occurs with 100% efficiency when an exciton encounters an open trap, the fluorescence will remain low until the very last reaction centre of PSII becomes closed, since every quantum of light absorbed by PSII will be efficiently trapped providing there is an open reaction centre somewhere within the pigment bed.

However, if transfer of excitation energy within PSII is less fast, or trapping is not 100% efficient, then fluorescence will increase slowly at first, increasing progressively more quickly as more reaction centres become closed. This leads to a pronounced sigmoidal shape of the fast fluorescence induction curve.

Experimentally, therefore, the shape of the O → P chlorophyll fluorescence induction curve can distinguish between these models of the PSII photosynthetic unit.

In fact, experiments first conducted by Joliot and Joliot[176] on dark adapted Chlorella showed that the fast, Q-dependent induction of fluorescence was sigmoidal in shape, as predicted by the statistical model of the PSU. However, the interpretation Joliot and Joliot placed on these results was based on a model of restricted energy transfer between PSII photosynthetic units, somewhat intermediate between the isolated, monocentral and statistical models. They proposed that each PSU was basically a separate entity containing a single reaction centre, but that a certain probability, p, existed of transfer of excitation energy from a closed PSU to an open neighbour. They derived the following relationship between ϕ_f and the proportion of closed units (1 - Q):

$$\phi_f = \frac{(1 - p)\ (1 - Q)}{1 - p\ (1 - Q)} \qquad \text{Eq. 1.12}$$

Such a model also predicts sigmoidal shapes of the O → P fluorescence induction, although it should be pointed out that other models involving discrete PSU's containing more than one trap with no transfer of excitation energy between units (isolated multicentral model) are also consistent with this data.

More recently Bennoun has found that the shape of the fast induction curve in Chlorella is variable depending on whether the algae are pre-illuminated to State I or State II.[177] In the relatively high fluorescing State I, induction was found to be sigmoidal, and a value of p computed to be 0.63 in agreement with the earlier results of Joliot and Joliot.[178] However, preillumination in red light which drives the algae into the low fluorescing State II condition resulted in a more exponential induction rise after a short dark period sufficient to reoxidise Q but not to allow the algae to return to State I. Bennoun concluded that a decrease in the probability of transfer of excitation energy between PSII photosynthetic units occurred during State I to State II transition. Applying the model of Joliot and Joliot, he found that p had decreased from 0.63 to 0.45.[177]

The same features are also shown by broken chloroplasts in cation-sensitive high and low fluorescing states.[157,170-172,174,175] In the presence of divalent cations (5 mM) or monovalent cations (100 mM) the O → P fluorescence induction is distinctly sigmoidal, whereas chloroplasts suspended only in monovalent cations at concentrations of a few mM (low fluorescence yield), the induction is very nearly exponential.

Thus kinetic studies of chlorophyll fluorescence provide another striking example of the similarities between State II → State I transition *in vivo* and the addition of cations to isolated broken chloroplasts initially suspended in media which result initially in the low fluorescing

state. It seems in both cases, that an increase in the quantal efficiency of PSII at the expense of PSI is also accompanied by an increase in the transfer of excitation energy between PSII units.

1.3 Other effects of cations on the primary photochemical reactions of broken chloroplasts

An increase in the distribution of light energy to PSII at the expense of PSI would be expected to be manifested in an increase in the quantal efficiency of photochemical reactions of PSII and a decrease in those of PSI. A good deal of evidence exists that this is indeed the case.

1.3.1. Partial reactions of photosynthesis

The use of artificial electron donors and acceptors together with appropriate inhibitors of non-cyclic electron transport allows electron flow to occur which utilises only portions of the complete electron transport pathway from H_2O to $NADP^+$. Under appropriate conditions, therefore, measurement of PSII dependent photochemical reactions can be made independently of the functioning of PSI and vice-versa. Use of such partial reactions allows clear-cut decisions to be made concerning the effects of cations on the quantum yield of photochemistry in PSII and PSI providing that low enough illumination is employed so that photochemical reactions are light limited.

Several workers have measured the relative quantum yield of PSII reactions (DCPIP or FeCy reduction) of chloroplasts suspended in low levels of monovalent cations, and nearly all agree that addition of Mg^{2+} (or high concentrations of monovalent cations) at concentrations which increase fluorescence yield also increase the quantum yield of PSII mediated photochemical reactions.[157,171,174,178,179] These results are consistent with the hypothesis that an overall increase in the quantum yield of PSII

is induced by cations. If this occurs at the expense of PSI, a decrease in the relative yield of PSI mediated reactions should also occur. The relevant experiments concerning this point have yielded conflicting results.

Using $DCPIPH_2$ and $NADP^+$ as artificial electron donor and acceptor of PSI respectively, Murata[157] and others[178] found a decrease in the rate of electron transport on addition of Mg^{2+} to broken chloroplasts. In contrast, Marsho and Kok[179] and Bose[180] found that Mg^{2+} stimulates the rate of $NADP^+$ reduction under similar conditions. However, Marsho and Kok also noted[179] as did Harnischfeger and Shavit[181] that $NADP^+$ reduction appeared to show an additional cation dependency which was not related to spillover effects. This was distinguished by a lower requirement for monovalent cations, and may be related to cation stimulation of ferredoxin activity. These authors have shown[179,181] that the rate of $NADP^+$ reduction is maximally stimulated in the presence of only 30 mM monovalent cations, and that further addition of divalent cations results in a decrease in electron transport consistent with lower excitation energy delivery to PSI. Methyl Viologen (MeVi) an artificial electron acceptor of PSI is not dependent on the activity of ferredoxin and the PSI dependent partial reaction involving this reagent is not stimulated on adding 30 mM monovalent cations to chloroplasts. However, divalent cations have been shown to markedly reduce the PSI dependent reduction of Methyl Viologen by $DCPIPH_2$ when chloroplasts are illuminated with light of wavelength less than 690 nm.[174,178,179] These results are also consistent with the hypothesis that cations decrease the distribution of light energy to PSII. However, one report has appeared[171] in which no effect of $MgCl_2$ on the $DCPIPH_2$/MeVi reaction was observed. These authors proposed that cations increase the quantum yield of PSII by inducing a decrease in the non-radiative dissipation of excitation energy (K_h) in PSII, a process which would not therefore affect the quantal efficiency of PSI (providing spillover was absent).

Also, Marsho and Kok[179] observed that cations decrease the quantal efficiency of a PSI dependent partial reaction in the presence of 650 nm, but not 710 nm light. The quantum yield of a PSII dependent partial reaction was increased by cations when light of 690 nm or 710 nm wavelengths were used, but not in 650 nm light. These results cannot be explained by changes either in α or in K_t.

Despite these discrepancies, the opinion put forward in this thesis is that the bulk of experimentalevidence qualitatively supports the hypothesis that cations exert effects both on the partial photochemical reactions of PSI and PSII and on fluorescence, by influencing the distribution of excitation energy between the two photosystems. However, the results of Jennings and Forti[171] and the more careful study of Marsho and Kok[179] indicate that additional cation effects may also occur which are at present, not at all well understood.

1.3.2. Non-cyclic electron flow involving both PSII and PSI

The effects of cations on the rate of non-cyclic electron transport reactions mediated by both PSII and PSI have also been studied.[179,180,182] However, the interpretation of these results as regards alterations in the distribution of excitation energy to PSI and PSII induced by cations requires care.

The rate of electron transport which utilises both photosystems will be optimal when PSI and PSII receive an equal share of the initial absorbed light energy. Under low salt conditions when spillover is prevalent, the rate of electron transport will be increased or decreased on adding cations depending on whether the illuminating conditions initially favour PSI or PSII. For example, if the light is initially absorbed by PSII more than PSI, sub-optimal rates of electron transport will be observed due to over

excitation of PSII relative to PSI. An inhibition of spillover on adding cations will inhibit electron transport even further by diverting even more light energy to the already over-excited PSII. In the presence of PSI light, however, redirection of some absorbed excitation energy to PSII at the expense of PSI may stimulate electron transport.

Marsho and Kok[179] have found that in 690 nm light, the rate of the $H_2O \rightarrow$ MeVi reaction was stimulated by divalent cations, whereas if 650 nm light was used, no change in overall rate was observed. These results can be explained if 690 nm light is preferentially absorbed by PSI under all conditions, but 650 nm light over-excites PSI in the absence of cations, and over-excites PSII in the presence of cations. No change in the rate of electron transport would therefore be observed in 650 nm light on adding cations to chloroplasts, since the effect would be to make PSI rate limiting instead of PSII. However, it must be pointed out that results of the effect of cations on partial reactions also given by those workers do not support this hypothesis on the distribution of 650 nm light to the two photosystems.[179] Other workers[180,182] found that divalent cations stimulate the rate of $NADP^+$ reduction coupled to water oxidation under any set of illumination conditions. However, as this stimulation may also include an increase in the activity of Ferredoxin (described in the previous Section), such results are difficult to evaluate as regards the effect of cations on spillover or changes in α.

Clearly, further careful work is required in this area of study before definite conclusions can be made concerning the effects of cations on overall rates of electron transport involving both PSI and PSII.

1.3.3. Enhancement

The observation of enhanced rates of non-cyclic electron transport on combining a light beam favouring PSII with one favouring PSI should depend

on the initial distribution of light energy between PSII to PSI. Measurement of enhancement in broken chloroplasts would therefore be expected to be dependent on the cation constitution of the suspending medium, and this has recently been shown to be the case. Using $NADP^+$ as electron acceptor, Sun and Sauer[183] and Sinclair[184] have found that in the presence of high levels of $MgCl_2$, the quantum yield of a PSII light is markedly increased by superimposing a second light beam favouring PSI. However, little enhancement of PSII light by PSI light was observed when chloroplasts are suspended in low levels of monovalent cations. These results have been confirmed by Marsho and Kok using Methyl Viologen as terminal electron acceptors.[179] These results are thus consistent with the hypothesis that, in the presence of low levels of monovalent cations, spillover of excitation energy from PSII to PSI equalises the initial imbalance of absorbed light. Alternatively, Sun and Sauer have proposed[183] that under all conditions, light is equally absorbed by PSII and PSI, but that divalent (5 mM) or monovalent (100 mM) cations promote spillover of excitation energy from PSI to PSII. Such a model qualitatively predicts similar experimental results as that of Murata where spillover is proposed to occur in the opposite direction and is inhibited by these concentrations of cations

Theoretically, however, the model of Sun and Sauer seems less likely for several reasons. Firstly, excitation energy transfer from PSI to PSII is energetically less favourable due to the predominantly shorter wavelength absorption of chlorophyll a in PSII compared to PSI. Secondly, Sun and Sauer propose that this spillover from PSI to PSII creates an imbalance of light energy distribution where previously, no such imbalance existed. This implies that excitation energy transferred to PSII cannot be passed back to PSI. On the basis of the Förster resonance energy transfer mechanism it is difficult to imagine how energy transfer from PSI to PSII can be unidirectional.

1.4. Spillover versus pigment shift models

Although discussed mainly in terms of Murata's model[157] of excitation energy spillover from PSII to PSI, the apparent redistribution of incoming light energy between the two photosystems both *in vivo* (by preillumination) and in broken chloroplasts (by cations) may also occur through spillover from PSI to PSII or changes in α, the fractional absorption of PSII.

These different models can be differentiated experimentally, though so far, this has mainly been carried out in regard to the effects of cations on isolated broken chloroplasts. To some extent, Murata[157] based his model on analysis of the area above the Q-dependent fast fluorescence induction curve in the high or low fluorescing state of broken chloroplasts pre-incubated with different concentrations of cations (see Section 1.2.6.3). According to this model, a change in α should alter the normalised areas (see earlier), whereas experimentally, large changes in the normalised areas were not found in the presence and absence of cations. Murata concluded therefore that changes in K_t, the rate constant for transfer of excitation energy from PSII to PSI were responsible for the fluorescence changes.

Support for this model also comes from measurements of fluorescence lifetime. An increase in one of the rate constants for dissipation of chlorophyll singlet excitation energy within PSII would be expected to result in a marked decrease in the lifetime of PSII chlorophyll fluorescence. However, a change in α or an increase in the spillover of excitation energy from PSI to PSII is equivalent only to changing the numbers of excitons within PSII and would not be expected to significantly affect PSII fluorescence lifetime. This can be seen from the following argument.

The intrinsic lifetime (τ_o) is the time required for half the excitation to dissipate if fluorescence was the only means of singlet state de-excitaton. Thus, assuming first order decay of excitation energy via fluorescence:

$$\tau_o = \frac{1}{K_f} \qquad \text{Eq. 1.10.}$$

However, in the thylakoid the actual lifetime (τ) is considerably shortened by the additional routes of exciton dissipation. Thus assuming the statistical model of the photosynthetic unit, τ, is given by equation 1.11:

$$\tau = \frac{1}{K_f + K_h + K_t + K_p\ (Q)} \qquad \text{Eq. 1.11.}$$

Combining equations 1.10 and 1.11, we find:

$$\frac{\tau}{\tau_o} = \frac{K_f}{K_f + K_h + K_t + K_p\ (Q)} \qquad \text{Eq. 1.12}$$

Combining equation 1.12 with equation 1.9 (see Section 1.2.6.3)

$$I_f = \alpha I_o \frac{\tau}{\tau_o} \qquad \text{Eq. 1.13}$$

Since τ_o is generally assumed to be constant (equal to 15.7 nsecs.) it can be seen from equations 1.12 and 1.13 that I_f will vary linearly with τ if changes in K_t occur. However, a change in α (or excitons arriving from PSI) will affect I_f, but not the lifetime of fluorescence decay.

The isolated, monocentral unit model of PSII also predicts a change in the mean lifetime of τ if changes in spillover from PSII to PSI occur, but in this case the relationship with fluorescence intensity is non-linear.[185]

Briantais et al have measured the lifetime of fluorescence in broken chloroplasts during the Q-dependent fluorescence induction period in both the cation-induced high and low fluorescing states.[174] In both situations, the fluorescence lifetime increased linearly with fluorescence yield throughout the induction phase. Furthermore, the increased overall fluorescence yield in the presence of divalent cations was associated with a lengthening of fluorescence lifetime. These results are consistent with significant changes in K_t or K_h induced by cations, but inconsistent with

changes in α or spillover from PSI to PSII as the sole cause of the fluorescence yield change. However, they do not rule out the possibility that these latter mechanisms may contribute to the changes in PSII fluorescence yield.

The results of Briantais et al on the linear relationship between fluorescence lifetime and yield during the induction period also suggest that significant energy transfer between PSII photosynthetic units occurs in chloroplasts in the high or low fluorescing state. These findings are not easy to reconcile with data from the shape of fluorescence induction curve. In thepresence of monovalent cations at low concentration (10 mM), this is nearexponential[174] indicating minimal transfer of excitons between PSII units (see Section 1.2.6.3).

Recently Moya[185] on reinvestigating this problem concluded that the relationship between ϕ_f and τ was not strictly linear, and therefore he suggested that energy transfer between PSII units was restricted. The cause of the discrepancy between these results is not clear. It is also pertinent to point out that these methods of measuring fluorescence lifetime involve correlation of the phase angle change between highly modulated actinic illumination and the resulting modulated fluorescence. Such methods suffer from the need to make many assumptions, one of which is that the decay of fluorescence in the dark is purely exponential function of time.

The greater time resolution of recently developed picosecond fluorimetric techniques allows direct analysis of the fluorescence decay profile after a short (10 psec.) flash of light. Although yet in infancy, these techniques already indicate that fluorescence decay is non exponential.[186] Further developments in this field should allow comprehensive studies in the time region of energy transfer and trapping within PSII, and considerably clarify our present rather poor understanding of these events.

Analysis of the fast fluorescence induction at low temperatures ($77^{o}K$) recently performed by Butler and Kitajima also supports the hypothesis that changes in K_t are predominantly involved in the cation induced redistribution of light energy between PSI and PSII.[126] Since PSI fluorescence has been shown to be insensitive to the rate of excitation energy trapping at the PSI reaction centres,[149] they argued that any variable fluorescence induction detected in the F_1 (PSI) emission at $77^{o}K$ must have arisen from excitation energy transferred from PSII. In fact, they found that metal cations at concentrations which increased room temperature fluorescence of isolated broken chloroplasts, decreased the variable component of PSI at fluorescence at $77^{o}K$. They therefore concluded that under these conditions, a decrease in K_t was elicited by addition of cations. Quantitative estimates, based on a rather complex model of energy transfer within PSII, indicated that the yield of spillover from PSII to PSI was decreased from 0.12 to 0.065 for open PSII traps, and 0.28 to 0.23 for closed PSII traps. The data also indicated that an increase in α of 9% had also to be included to quantitatively account for the fluorescence yield changes.[128]

In conclusion it appears that the increase in fluorescence yield of broken chloroplasts brought about by cations involves mainly a redistribution of excitation energy in favour of PSII at the expense of PSI. This may occur both by a decrease in spillover and an increase in the initial fractional absorption of incident light by PSII.

In vivo, the State I/II transition also may involve changes in spillover or initial absorption of excitation energy between the two photosystems. Unfortunately, neither fluorescence lifetime measurements nor low temperature fluorescence induction analyses have been performed on algae preilluminated to State I and State II.

Wang and Myers[187] measured the oxygen yield after a short non-saturating flash given to algae preilluminated to either State I or State II. They

found a significant increase in the quantum yield of O_2 evolution of PSII in State I as compared to State II. Such an increase in quantum yield rules out an earlier suggestion by Delrieu[188] that State I/II changes are due to a change in the apparent equilibrium constant for reversible electron flow between the primary acceptor of PSII and the electron donor to PSI. Wang and Myers concluded that their results could be explained by changes in α.[187] However, there seems no reason why changes in K_t, the spillover between PSII and PSI might not also contribute to the observed changes in PSII quantum yield. Further work on State I/II changes *in vivo* is required to choose between these possibilities.

1.5. Involvement of cations in State I/II changes *in vivo*

Whether cations are proposed to affect spillover or the fractional absorption of PSII in broken chloroplasts, the various similarities between such cation induced changes and those which occur during State I/II *in vivo* cannot be ignored. In Table 1.1., I have listed similarities some of which have been discussed in previous sections and a few which have not been mentioned in the text. The obvious similarities between the two processes suggests that State I/II changes may be brought about *in vivo* by an alteration in the cation levels within the *in vivo* chloroplast. As discussed in Section 1.1.6., this may occur by changes in the partition of cations between the intrathylakoid and stromal spaces of the intact chloroplast in response to development of a transthylakoid pH gradient.

The work presented in this thesis has been aimed at investigating the feasibility of this hypothesis, and several major questions have been considered:

1. What is the nature and direction of ion fluxes in response to light induced proton pumping into the intrathylakoid space *in vivo*? This has been investigated in isolated intact chloroplasts retaining fun-

TABLE 1.1.

Similarities between addition of low levels of divalent cations (2-5 mM) or high levels of monovalent cations (100 mM) to broken chloroplasts[a] and the State II to State I transition *in vivo*.

	Observation	Addition of cations to broken chloroplasts	State II to State I *in vivo*
1.	Fluorescence yield at room temperature	Increased[157,168-172]	Increased[155,156]
2.	Ratio: PSII:PSI fluorescence at low temperature (77°K)	Increased[128,157,169,194]	Increased[156]
3.	Fast fluorescence induction	More sigmoidal[157,174,175,177]	More sigmoidal[177]
4.	Transfer of excitons between PSII units (monitored via O_2 evolution)	Increased[177]	Increased[187]
5.	Enhancement	Increased[183,184]	Increased[155,188]
6.	Quantum yield of PSII dependent O_2 evolution	Increased[157,171,178,179]	Increased[177,187]
7.	Rate of dark deactivation of O_2 precursor in water splitting enzyme	Accelerated[177]	Accelerated[177]
8.	Thylakoid stacking	Increased[223,224]	Increased[177] No change[192,242]

(a) Chloroplasts initially suspended in media containing low levels of monovalent cations (2-10 mM).

ctional outer envelopes by studies involving the use of chlorophyll *a* fluorescence as an intrinsic probe to monitor ion fluxes within the intact organelle.

2. Where are the sites of action of cations located on the thylakoid membrane? The 'sidedness' of the thylakoid membrane becomes an important consideration when relating the role of ion fluxes *in vivo* to the mechanism responsible for State I/II changes. For example, as visualised in Fig. 1.3, proton uptake into the thylakoid may result in a decrease in the levels of cations in the intrathylakoid space, and an increase in cation concentration of the stroma. Cation controlled spillover may therefore be increased or decreased depending on whether cations act predominantly on the inner or outer surfaces of the thylakoid membrane. Little attention to this problem has been paid in the past.
3. What is the relationship between cation sensitive changes and high energy state induced quenching of fluorescence yield in broken chloroplasts? In the past these two types of fluorescence changes have been treated as unrelated phenomena when studied in broken chloroplasts. On the other hand, an intimate relationship would be expected if the generation of cation fluxes in response to hydrogen ion gradients were responsible for State I/II changes *in vivo*. This problem has been re-investigated in Section 3 of this thesis where it will be shown that the two phenomena are, in fact, closely related.
4. What is the physical mechanism by which cations interact with the thylakoid membrane? Cation induced changes in the distribution of excitation energy between PSI and PSII must involve structural changes in the thylakoid membrane which somehow alter the arrangement of chlorophyll *a* within the membrane. The way in which cations interact with the thylakoid membrane has been carefully investigated through studies of cation sensitive changes in chlorophyll *a* fluorescence and a novel and

quantitative model concerning the effects of cations on fluorescence and chloroplast ultrastructure will be presented in Section 4.

The results of experiments designed to answer these four (and other) questions will be described in Sections 3 and 4. It will be concluded that light induced fluxes of cations, and especially of Mg^{2+}, may well be important in mechanisms *in vivo* which serve to optimise the efficiency of photosynthesis in green plants under differing illumination conditions.

SECTION 2

MATERIALS AND METHODS

2.1. Isolation of chloroplasts

2.1.1. Intact chloroplasts

Intact chloroplasts (those retaining functional outer envelopes) were routinely isolated from market spinach or pea seedlings (var. Feltham First) by the method of Stokes and Walker.[189] Whole, undamaged spinach leaves were illuminated for 30 min. in ice cold water, then rinsed with distilled water prior to maceration. Pea seedlings grown in vermiculite without nutrients in growth chambers employing 12 hr. light/dark cycles (20°C) were harvested after 1-3 hr. of illumination between the 12th and 15th day.

80 g of deveined spinach leaves or pea seedlings were briefly macerated (2-5 secs.) using a Polytron homogeniser in 200 ml. of semi frozen isolation medium consisting of 0.33 M sorbitol, 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 5 mM $MgCl_2$, 4.3 mM NaCl (0.1% W/V) and 2.3 mM Na isoascorbate (0.2% W/V), pH 6.5. The macerate was squeezed through two layers of muslin, and then allowed to filter through eight layers of muslin in which was sandwiched a thin layer of cotton wool. This procedure removed leaf fragments and large cell debris from the resulting suspension of intact and broken chloroplasts and other cell organelles. The intact chloroplast fraction was then isolated by a rapid centrifugation step (within 55 secs.), using an MSE Super Minor bench centrifuge accelerated rapidly to 2,400 xg and braked by hand. The resulting pellet also contained some broken chloroplasts distinguished as a loose upper pellet layer, and these were partially removed by gentle agitation and decantation. The final pellet (intact chloroplast) was resuspended in a small volume of one or two media: (i) assay medium, containing 0.33 M sorbitol, 1 mM $MgCl_2$ 1 mM $MnCl_2$, 2 mM EDTA, 50 mM N-2

hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES)/KOH pH 7.6; or (ii) low salt buffer consisting of 0.33 M sorbitol, 10 mM HEPES and sufficient tris(hydroxymethyl) aminomethane (tris base) to bring the medium to pH 7.6 (approx. 7.5 mM tris). All steps during chloroplast isolation were performed as close as possible to 0-4°C, and the whole procedure completed within five minutes of first macerating the leaves. The resulting stock suspensions of intact chloroplasts were maintained at 0-4°C (on ice) at concentrations of 1-5 mg. chlorophyll ml^{-1} as determined by the method of Arnon.[190] Experiments with intact chloroplasts were performed after dilution of this stock in either assay medium or low salt buffer as required.

All such preparations of intact chloroplasts in fact consisted of heterogeneous mixtures which included some broken chloroplasts (i.e. those from which the outer envelope had been removed or seriously damaged). The proportion of intact chloroplasts ('% intactness') was estimated by the method of Heber and Santarius.[103] This method depends on the impermeability of the chloroplast outer envelope to ferricyanide. By comparing the rates of ferricyanide-dependent oxygen evolution (electron transport) by intact and osmotically-shocked broken chloroplasts under identical conditions, the proportion of intact organelles in any one preparation was deduced. This was found to vary from 30 to 80%, and seemed to depend on the quality of leaves from which intact chloroplasts were isolated.

2.1.2. Broken chloroplasts

Broken chloroplasts were routinely prepared from intact organelles by removal of the outer envelopes of those latter preparations. This was performed according to two protocols:

i. For all of the experiments with broken chloroplasts reported in

Section 3, intact chloroplasts were ruptured by brief osmotic shock in water (less than 15 seconds) followed by addition of an equal volume of double strength assay medium or low salt buffer. This relatively mild method of preparing broken chloroplasts (performed in the measuring cuvette immediately prior to experimentation) gave rise to thylakoids whose properties seem to approximate more closely to those of thylakoids *in vivo* than somewhat harsher techniques involving longer exposure times of the membranes to non-physiological media (see, for example, Ref.19).

ii. In order to prepare broken chloroplasts free of excess cations for experiments conducted at low ionic strength (Section 4) another procedure based on that of Gross[191] was developed. Intact chloroplasts were prepared and immediately ruptured by suspension in 15 mls of water for two minutes. Where indicated, 0.5 mM EDTA (pH 7.0 with NaOH) was also present ('EDTA-washed chloroplasts'). This was followed by addition of 15 ml of a double strength 'cation-free medium' (0.1 M sorbitol, with sufficient tris base (0.1-0.2 mM) to bring the final pH to 7.0) and the suspension allowed to stand for a further three minutes. Shocked, washed chloroplasts largely free of monovalent cations were then collected by centrifugation at 6,000 x g for four minutes, and resuspended in a minimal volume of cation-free medium without further additions.

For the fluorescence measurements described in Sections 2.2, and 2.3, chloroplasts were suspended at 5-30 µg chlorophyll ml^{-1} in a 10 x 10 mm glass cuvette (reaction volume 3 ml) with assay medium, low salt buffer or cation-free medium as required.

2.2. Measurement of chlorophyll a fluorescence

2.2.1. Relative yield in continuous illumination

In most of the experiments reported in this thesis, chlorophyll a fluorescence was detected at right angles to a single beam of constant intensity which also served as an actinic light for photosynthetic reactions. Any changes in fluorescence emission intensity with time were therefore related to changes in fluorescence yield of chlorophyll a in the thylakoid. Incident light from a 200 Watt tungsten projector lamp was transmitted through a filter combination of Balzer Calflex C, 2 mm Schott BG 18 and 2 mm Schott BG 38 (or alternatively, Balzer Calflex C and 4 mm Schott BG 18) which gave rise to broad band blue illumination at an intensity of 70-80 Wm^{-2} at the cuvette surface. Chlorophyll a fluorescence at 90^{o} to the actinic light was detected by an EMI 9958 B (S-20 response) photomultiplier screened by a Balzer B 40 682 nm, 685 nm or 695 nm interference filter together with appropriate Schott red cut-off filters to eliminate scattered actinic light. The photomultiplier output was directly displayed on a Rikadenki chart recorder without further preamplification. Small volumes of reagents (100 µl) were added to the cuvette during the course of an experiment by injection through light-tight diaphragms with microsyringes. Mixing artifacts were checked by the appropriate controls and alcohol added did not exceed 1% of the final reaction medium.

2.2.2. Light intensity curves

In order to measure the intensity dependence of light induced chlorophyll a fluorescence quenching in intact chloroplasts, the methods outlined in previous sections were refined in the following way. The blue excitation light was modulated by inserting a Brookdeal model 9479 light chopper between the lamp and the blue excitation filter com-

bination. Modulated fluorescence excited by the chopped light was detected with the aid of a Brookdeal model 9401 lock-in amplifier. The intensity of the modulated (denoted as m) light was reduced to 0.5 Wm^{-2}. This low intensity ensured that this light induced very little actinic effects on photosynthetic reactions and served only as a measuring beam probing the relative fluorescence yield at any time. Photosynthetic reactions were brought about by a second unmodulated light beam (d.c. light) of similar wavelength to the measuring beam, angled at 45^{o} to the measuring beam and photomultiplier. The fluorescence excited by the d.c. beam was not detected by the lock-in amplifier. Any change in the emission intensity of modulated fluorescence could therefore be related to overall changes in fluorescence yield induced by the actinic d.c. light.

2.2.3. Enhancement effects of PSI light on chlorophyll fluorescence

Chlorophyll fluorescence was excited by a modulated blue light of intensity $\sim$3 Wm^{-2} as described in Section 2.2.2. This intensity was sufficient to cause overall reduction of a portion of Q. Enhancement of electron transport on superimposing a non-modulated PSI light (Balzer B40 705 nm interference plus 2 mm Schott 695 nm red cut-off filters to give intensity of 4 Wm^{-2}) was detected as a rapid decrease in the yield of modulated fluorescence around 730 nm (Balzer B40 731 nm interference plus 2 mm Schott RG 715 cut-off filters).

2.2.4. Fluorescence emission spectra at liquid nitrogen temperatures ($\sim 77^{o}K$)

For measurement of low temperature emission spectra, chloroplasts were diluted to 10 µg ml^{-1} in assay medium and pretreated with appropriate reagents. Samples of 0.1 ml of suspension were then taken up into cylindrical quartz tubes and either maintained in the dark or illuminated

in the apparatus described in Section 2.2.1. for three minutes. After this period, samples were rapidly frozen (less than 5 seconds) in liquid nitrogen. Where necessary preillumination was continued throughout the freezing procedure. The quartz tubes were kept frozen in a clear Dewar flask containing liquid N_2, and chlorophyll fluorescence emission spectra measured using a Perkin-Elmer MPF-4 spectrofluorimeter equipped with an R446 F red-sensitive photomultiplier. Samples were excited with monochromatic light at 435 nm (slit width 20 nm) at sufficient intensity to bring about complete reduction of Q, the primary electron acceptor of PSII at these temperatures. Emission spectra were scanned at 60 nm Min^{-1} with a motor driven monochromator set at 5 nm slit width. Spectra were not corrected for the sensitivity of the monochromator/photomultiplier combination at different wavelengths.

The relative yield of fluorescence as judged from the peak heights of emission bands at 77°K was seen to vary between duplicate samples of identical pre-treatment. This variation could be ascribed to differences in the size and shape of the quartz sample tubes.[192] For this reason, only the ratios of PSII fluorescence (F685 and F695) to PSI fluorescence (F735) within each sample (which did not vary more than 10% between duplicates) was used to compare the effect of different pretreatments on 77°K emission characteristics.

2.3. 9-amino acridene (9-AA) fluorescence measurements

2.3.1. Binding of 9-AA to the thylakoid membrane

Changes in the binding of 9-AA to the thylakoid brought about by altering the concentration of cations in the reaction medium were measured as changes in the fluorescence yield of 9-AA as described by Bose.[180] The same technique described in Section 2.2.1. was used for this purpose with the following modifications. 9-AA fluorescence was excited by light

filtered through Balzer Calflex C, 2 mm Schott BG38 and Balzer B40 398 nm interference filters (intensity 0.1 Wm^{-2}) and detected after transmission through a Balzer B40 495 nm interference filter. It was found using these filter combinations that some scattered actinic light reached the photomultiplier. The scattering artifact was quantitatively estimated from the apparent fluorescence detected from a reaction medium from which 9-AA had been omitted, and found to be 5% or less of the final signal in the presence of this fluorescent probe at concentrations of 20 µM.

2.3.2. High energy state quenching of 9-AA fluorescence

9-AA fluorescence was detected at right angles to a modulated (110 hz) excitation light (0.1 Wm^{-2}) with the aid of a Brookdeal model 9401 lock-in amplifier. The same filter combinations described in Section 2.3.1 were used to filter excitation light from a 200 W projector source and to screen the photomultiplier (EMI 9558B) from stray excitation light. The intensity of the measuring beam was too weak to effect appreciable electron-transport in broken chloroplasts. A second, unmodulated light beam (Balzer Calflex C, 2 mm Schott BG18, 2 mm Schott BG38 to give intensity 50 Wm^{-2}) at 180° to the measuring beam was used as actinic (d.c.) light. 9-AA fluorescence decreases detected by the lock-in amplifier in response to actinic illumination were reversed by uncouplers and therefore concluded to be due to development of the chloroplast high energy state and not to any direct effects of the unmodulated light on the detection system. Chlorophyll a fluorescence was also measured simultaneously on this apparatus using a second photomultiplier screened with Balzer's 731 interference and 2 mm Schott 715 nm cut-off filters. The sensitivity of the photomultiplier was adjusted so that only chlorophyll fluorescence excited by the actinic light, and not the modulated beam were detectable.

The concentration of 9-AA used in the reaction cuvette was 3.3 µM.

2.4. Absorption spectra

The absorption spectra of DAD suspended in low salt buffer at pH 7.6 or 0.33 M sorbitol containing 5 mM HEPES, 5 mM succinic acid adjusted to pH 4.5 with tris base were measured on an Aminco-Chance DW2 spectro fluorimeter in the split beam mode (slit width 5 nm). The reference cuvette contained buffer only. DAD was chemically oxidised by excess ferricyanide, and the small contribution of ferricyanide to the resulting DAD spectrum subtracted by comparison with the spectrum obtained with ferricyanide alone. The maximall extinction coefficient (ε_{max}) for oxidised DAD absorption at 478 nm was determined from the average of several experiments at different concentrations of DAD by applying the Beer-Lambert law, Equation 2.1.

$$O.D. = \varepsilon_{max}\, c.l. \qquad \text{Eq. 2.1.}$$

Where O.D. was the optical density, c the concentration of DAD and l the pathlength of the reaction cuvette which was 10 mm. For the determination of ε_{max}, the optical density of oxidised DAD, in this case was measured with a SP600 spectrophotometer.

2.5. Measurement of electron transport

Electron transport was measured as oxygen evolution (or O_2 uptake when methyl viologen was used as electron acceptor) with a Clarke-type oxygen electrode (Rank Bros. Cambridge). The reaction vessel contained chloroplasts at 50 µg chlorophyll ml^{-1} in a vigorously stirred reaction volume of 2 or 3 mls maintained at a temperature of $21 \pm 1^{o}C$. In most cases, red illumination obtained from 200 W projector source with a filter transmitting light of wavelength longer than 610 nm was used at an intensity of 70-80 Wm^{-2}. For experiments with poly-L-lysine red illumination at 15 Wm^{-2} obtained with 2 mm Balzer K6 interference filter was used to

induce electron transport. Rates of electron transport were detected as the rate of change of O_2 concentration in the medium and displayed on a Rikadenki chart recorder.

2.6. Measurement of pH changes

Changes in the pH of the suspending medium on illuminating broken chloroplasts contained in the reaction cell of a Rank O_2 electrode were detected using a combination glass electrode (Pye Unicam EJ702) inserted through the lid of the reaction vessel. Chloroplasts (at a concentration of 38 µg chlorophyll ml^{-1}) were suspended in low salt buffer containing 10 mM KCl and in which the concentration of HEPES/tris was reduced to 1 mM Illumination consisted of broad band red actinic light at 70-80 Wm^{-2}. Changes in pH on illumination were detected with a Pye model 290 pH meter and displayed on a Rikadenki chart recorder. The number of protons taken up by chloroplasts was estimated from the light/dark ΔpH changes calibrated by adding small aliquots of 10 mM HCl or 10mM NaOH. The initial pH of the medium was adjusted to pH 6.5 or pH 7.5 after equilibration with any reagents added as small aliquots (less than 100 µl) with microsyringes.

2.7. Reagents

Unless stated, all reagents used were obtained commercially and were of the highest available purity. Ionophore A23187 was a gift from Eli Lilly, Indianapolis (Lot.no. 361-066-275 and 361-V02-228) as were nigericin (Lot.no. 189-380-B-171a) and valinomycin (Lot.no. 488-833B-118-2). Beauvaricin was a gift from Dr R. Prince and originated from Dr R. Roeska, Indiana University.[193] Diaminodurene (DAD) was purchased from Eastman Kodak, or Aldrich Chemical Co. Similar results were obtained with DAD from either source although some differences were noted in the solubility

of this reagent in 50% ethanol/water. Fresh stocks of DAD (25mM) were prepared in 50% methanol/water no longer than 1 hour before experimentation.

Unpurified ferredoxin (also known as photosynthetic pyridene nucleotide reductase or PPNR) was isolated from spinach by the following procedure. 100 g of deveined leaves were thoroughly homogenised in 200 ml of ice cold buffer containing 20 mM tris/HCl, pH 8.0 with an MSE Atomix blender. The homogenate was filtered through muslin and exhaustively centrifuged at 6,000 xg to remove all but the smallest fragments of cell debris and chloroplast lamellae. The green supernatent was then applied to 10 x 100 mm column of DEAE cellulose (DE23) pre-equilibrated with 20 mM tris/HCl pH 8.0. Ferredoxin (brown coloured band) was then eluted from the column with a minimal volume (2.5 mls) of 20 mM tris/HCl pH 8.0 containing 0.8 M KCl. The ferredoxin fraction was then dialysed for 24 hours against a large volume of 20 mM tris/HCl pH 8.0 to remove KCl and used for experimentation without further purification.

SECTION 3

THE ROLE OF METAL CATIONS IN SLOW CHLOROPHYLL a FLUORESCENCE YIELD CHANGES IN ISOLATED INTACT AND BROKEN CHLOROPLASTS

3.1. Introduction

Studies with intact chloroplasts have recently provided evidence that light-induced cation fluxes may play a role in the regulation of various aspects of primary and secondary photosynthetic reactions. In this Section, I will consider the influence of such ion fluxes on the primary photochemical reactions.

Independently, Barber and Telfer,[194] and Krause[195,196] discovered that illumination of dark adapted, isolated intact chloroplasts resulted in slow fluorescence induction phenomena similar to those observed in the leaf. The general features of these slow changes in fluorescence yield are shown in Figure 3.1 (after Barber and Telfer). At the high light intensity used, Q[136] the primary electron acceptor of PSII was largely reduced (and PSII traps consequently closed) within a few seconds of illumination. This resulted in a high initial chlorophyll a fluorescence yield. The subsequent rate of slow fluorescence quenching ($t_{\frac{1}{2}} \sim 5$ mins in the presence of CO_2 only) was increased on adding physiological electron acceptors identical with or related to intermediates of the Calvin cycle. The light induced decline in fluorescence might therefore have been due to the build up of the prephosphorylation high energy state which depends on the rate of coupled electron transport, or to the re-oxidation of Q as electron transport slowly attained maximal rates in the light. Experiments with uncouplers such as nigericin, which facilitate H^+/K^+ exchange across thylakoid membrane indicated that fluorescence quenching was due to the formation of the ΔpH gradient within the intact

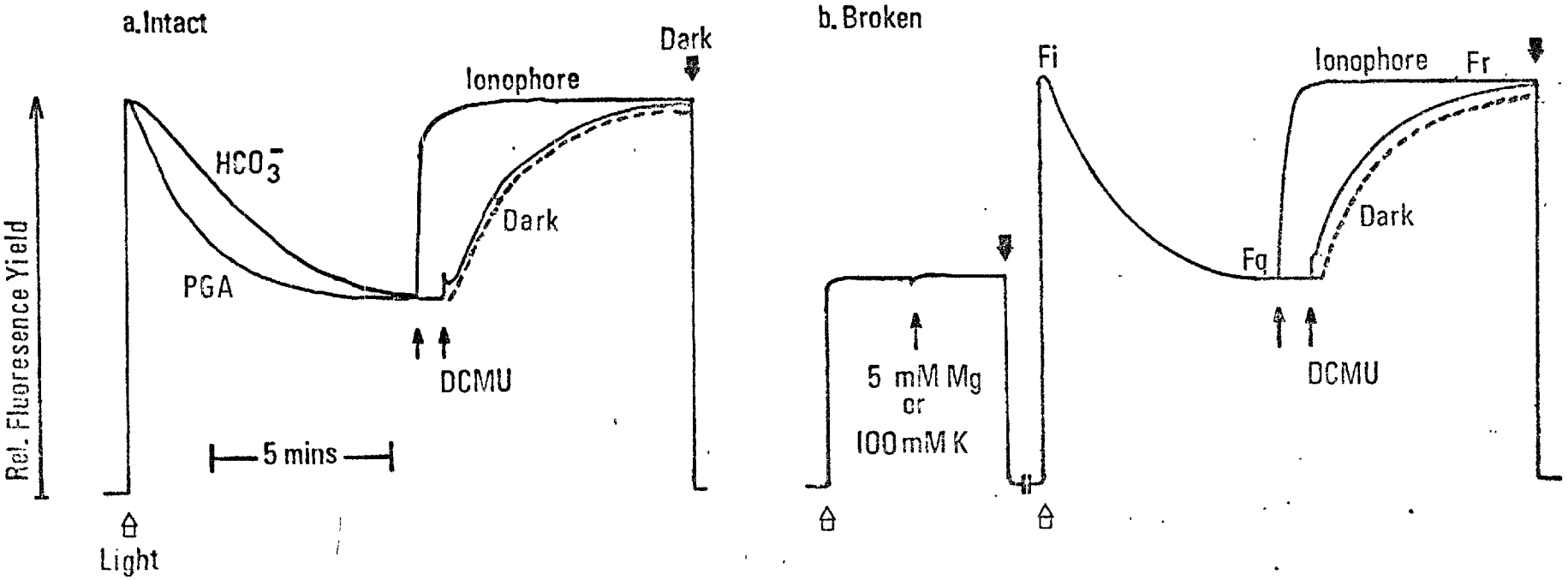


Fig. 3.1 An illustration of some of the slow chlorophyll fluorescence yield changes observed on illuminating a suspension of dark pretreated intact and broken chloroplasts suspended in assay medium: (a) intact chloroplasts (30 µg chlorophyll ml^{-1}) in the prescence of 5mM HCO_3 or 1mM PGA; (b) broken chloroplasts obtained by osmotically shocking intact chloroplasts (see Section 2.1.2). Additions: 0.1 µM nigericin or 2 µM A23187 (denoted ionophore) and 3.3 µM DCMU. Open arrow, light on; closed arrow light off (see Section 2.2.1 for other experimental details).

chloroplast. Nigericin was found to both reverse and inhibit such fluorescence quenching in a manner which was independent of the nature of the electron acceptor used.[194] In the presence of CO_2 or 3-phosphoglycerate (PGA) which require ATP for their reduction, nigericin reversed fluorescence quenching at similar concentrations known to dissipate the proton gradient. This nigericin-induced increase of the quenched fluorescence level might have been due to inhibition of electron transport which resulted from lack of ATP available for CO_2 or PGA reduction. An inhibition of electron transport would result in reduction of Q and an increase in fluorescence yield.

However, similar results were observed when oxaloacetate (OAA) was used as electron acceptor. OAA does not require ATP for its reduction. Uncoupling of OAA-dependent electron transport resulted in increased rates of OAA-dependent O_2 evolution and might have been expected to decrease fluorescence through Q effects. However, since nigericin increased fluorescence from the quenched level in the presence of OAA, the quenching in intact chloroplasts was clearly more related to the establishment of the high energy state within these organelles, than to Q effects.[194]

This conclusion is supported by simultaneous studies of chlorophyll a fluorescence yield and delayed light emission at 1 msec (msec DLE). DLE is thought to result from a back reaction of the primary products Z^+ and Q^- of excitation energy trapping within the PSII reaction centre.[72] It has previously been established that the formation of the chloroplast high energy state stimulates msec DLE,[72,87,197] probably by decreasing the activation energy for this back reaction.[72-75,175,198] Telfer and Barber (see Ref. 199) found that nigericin both reversed chlorophyll a fluorescence quenching and inhibited msec DLE in illuminated intact chloroplasts. Furthermore, the kinetics of fluorescence reversal and

msec DLE inhibition appeared to be logarithmically related. This would indicate that light-induced fluorescence lowering is directly related to the magnitude of the high energy state.[199] The antibiotic valinomycin, which may decrease the $\Delta\psi$ component of the electrochemical proton gradient when potassium ions are out of equilibrium, was observed to induce little reversal of slow fluorescence quenching. It was therefore concluded that light-dependent fluorescence lowering in intact chloroplasts was due solely to the build up of the chemical (ΔpH) component of the trans-thylakoid high energy state.[199]

Krause[196] also reached this conclusion from comparison of fluorescence quenching with increases in 180^{o} light scattering observed as an apparent absorbance change at 540 nm. Close kinetic correlation between these two phenomena was observed both in the leaf and in intact chloroplasts.[195,196] This observation led Krause to propose that both chlorophyll fluorescence and light scattering monitored structural changes in the thylakoid membrane brought about by proton uptake into the intrathylakoid space. However, as pointed out by this author and discussed in Section 4, it seems unlikely that the conformational changes responsible for these two effects are identical in each case even though both appear to reflect energisation of the membrane.

A period of darkness, or addition of DCMU in the light also restored quenched fluorescence of intact isolated chloroplasts to the high initial yield, but with markedly slower kinetics than those observed on addition of ionophores.[194] Apart from the small rapid phase on addition of DCMU (which presumably reflects rapid closure of the few PSII traps remaining open), these slower kinetics may reflect the dissipation of the proton gradient on terminating non-cyclic electron flow.

Subjection of intact chloroplasts to an osmotic shock (which results in disruption and removal of the outer envelope) resulted in a lowered

initial fluorescence (in assay medium containing only low activities of monovalent cations) and loss of the slow fluorescence induction phenomena.[194,196] However, addition of 6 mM Mg^{2+} (or Ca^{2+}) or higher levels of monovalent cations (> 100 mM) to such broken chloroplasts in the dark not only restored the high initial yield but also the slow subsequent fluorescence quenching. As shown in Figure 3.1, these reconstituted fluorescence changes in broken chloroplasts were very similar to those observed with intact organelles. In this case, fluorescence quenching appeared to be due to establishment of a pH gradient across thylakoids which was linked to slow, endogenous electron transport of the Mehler type[200] (reduction of O_2 by PSI, probably to the superoxide anion[201]).

The slow decrease in chlorophyll fluorescence which occurs during induction therefore appears to involve both metal cations and the establishment of the high energy state. It was concluded[194,196] that this process reflected displacement of metal cations from sites on the inner side of the thylakoid membrane in response to proton uptake into the intrathylakoid space. This was supported by observations that addition of cations to broken chloroplasts preilluminated to establish a significant ΔpH gradient across the thylakoid did not result in the usual cation induced increase in fluorescence until the pH gradient was dissipated by DCMU, uncoupling or darkening of the preparation.

Such light driven cation fluxes may be important in vivo in the mechanism controlling light distribution between PSI and PSII.[155] Experiments reported in this Section were designed to elucidate the nature of light dependent cation transport within intact chloroplasts and the involvement of such fluxes in State I/II changes. In addition, high energy state quenching of fluorescence brought about by cofactors which stimulate cyclic electron transport was reinvestigated in view of a possible contribution of cation fluxes to this type of fluorescence change.

3.2. Results

3.2.1. Light intensity curves

A number of authors have shown that in broken chloroplasts treated with DCMU to eliminate changes in Q, cations affect mainly the variable portion of chlorophyll a fluorescence yield and induce little change in the constant yield (F_o).[128,157,171,174,175] If slow fluorescence quenching observed in intact chloroplasts is due to removal of cations from their site of action in response to proton pumping, then this should also result in changes only in the variable fluorescence yield.

The results presented in Figure 3.2 show that this is indeed the case. In this experiment, relative chlorophyll a fluorescence of intact chloroplasts in the presence of PGA as electron acceptor was detected with a modulated light (denoted as m, see Section 2). The low intensity of this beam (0.5 Wm^{-2}) ensured that little overall reduction of Q occurred, and so the initial level of fluorescence obtained consists almost entirely of the constant yield component, F_o. It can be seen from Figure 3.2 that addition of an unmodulated light of much higher intensity (80 Wm^{-2}, denoted d.c. or actinic light) resulted in a three-fold increase in modulated fluorescence within the response time of the apparatus (< 10 seconds). Since unmodulated fluorescence excited by the d.c. beam was not detected by the lock-in amplifier, the increase in modulated fluorescence must be due to an overall increase in chlorophyll fluorescence yield. This is caused by rapid reduction of Q and closing of PSII traps in high actinic light intensities. The ratio of fluorescence levels in the presence and absence of strong d.c. light ($\frac{d.c.}{m}$) therefore provides a convenient measure of variable fluorescence induced by the actinic beam.

It can be seen from Figure 3.2 that continued illumination of intact chloroplasts in the presence of strong d.c. light resulted in slow

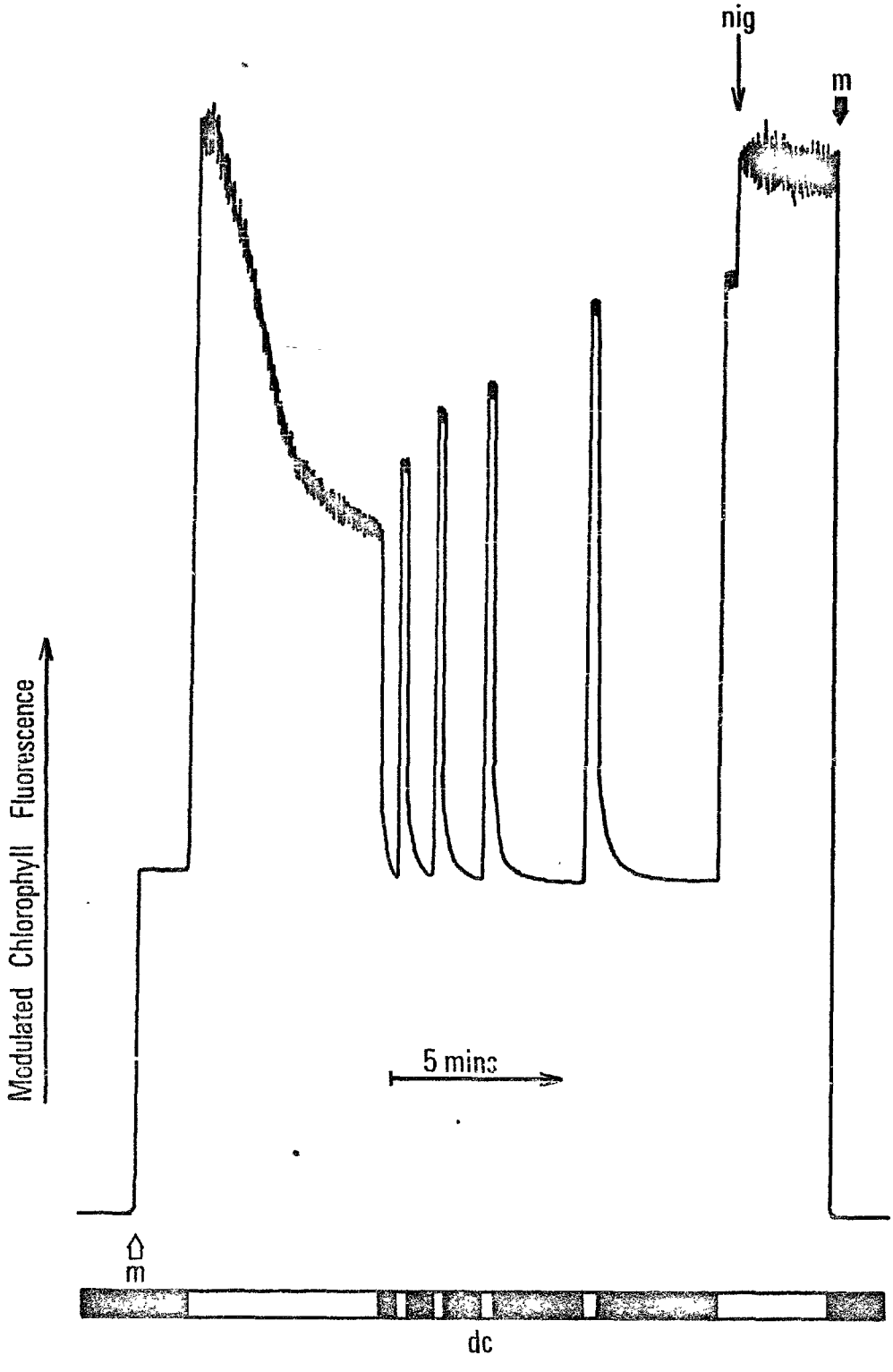


Fig. 3.2 Effect of high intensity d.c. light on modulated fluorescence excited by a weak modulated beam. Intact chloroplasts (65% intact by the ferricyanide estimation, see Section 2.1.1) were suspended in assay medium to a final chlorophyll concentration of 15 µg ml^{-1} in the prescence of 1mM PGA. Open arrow, modulated light on; closed arrow, modulated light off; open lower bar, d.c. light on; closed lower bar, d.c. light off (see Section 2.2.2 for further details).

quenching of chlorophyll fluorescence ($t_{\frac{1}{2}} \sim 2$ min) due to build up of the high energy state of thylakoid as previously reported by Krause[196] and Barber and Telfer.[194] On terminating d.c. illumination, the yield of modulated fluorescence rapidly decreased to much the same level observed before actinic illumination. This rapid decrease was due to reoxidation of Q and was speeded up on the presence of PSI light (results not shown).

Short periods (seconds) of strong actinic light given at variable times after termination of continuous d.c. illumination were used to monitor the recovery of high energy state quenched variable fluorescence to the high yield in the dark. It is assumed that the weak modulated measuring light is equivalent to darkness. As seen from Figure 3.2, even after ten minutes of darkness, the reversal of slow light-induced fluorescence quenching was not complete. Addition of nigericin, however, rapidly restored variable fluorescence to the maximum yield. Although large changes in the yield of variable fluorescence occurred both in the d.c. light and in the dark, little change occurred in the steady state F_o level observed in the presence of the measuring beam alone. The results clearly show that slow fluorescence changes of isolated intact chloroplasts due to build up and dissipation of the transthylakoid high energy state, affect only the variable portion of chlorophyll fluorescence. It is also clear that such changes are considerably slower than fluorescence changes due to the redox state of the PSII traps on commencement and termination of actinic illumination. Hipkins[175] took advantage of this fact to measure the Q-dependent fast fluorescence induction at the initial and light quenched levels. He also concluded that the high energy state only affects the variable portion of yield.

Figure 3.3 shows the effect of varying intensities of d.c. illumination on some of the fluorescence changes depicted in Figure 3.2

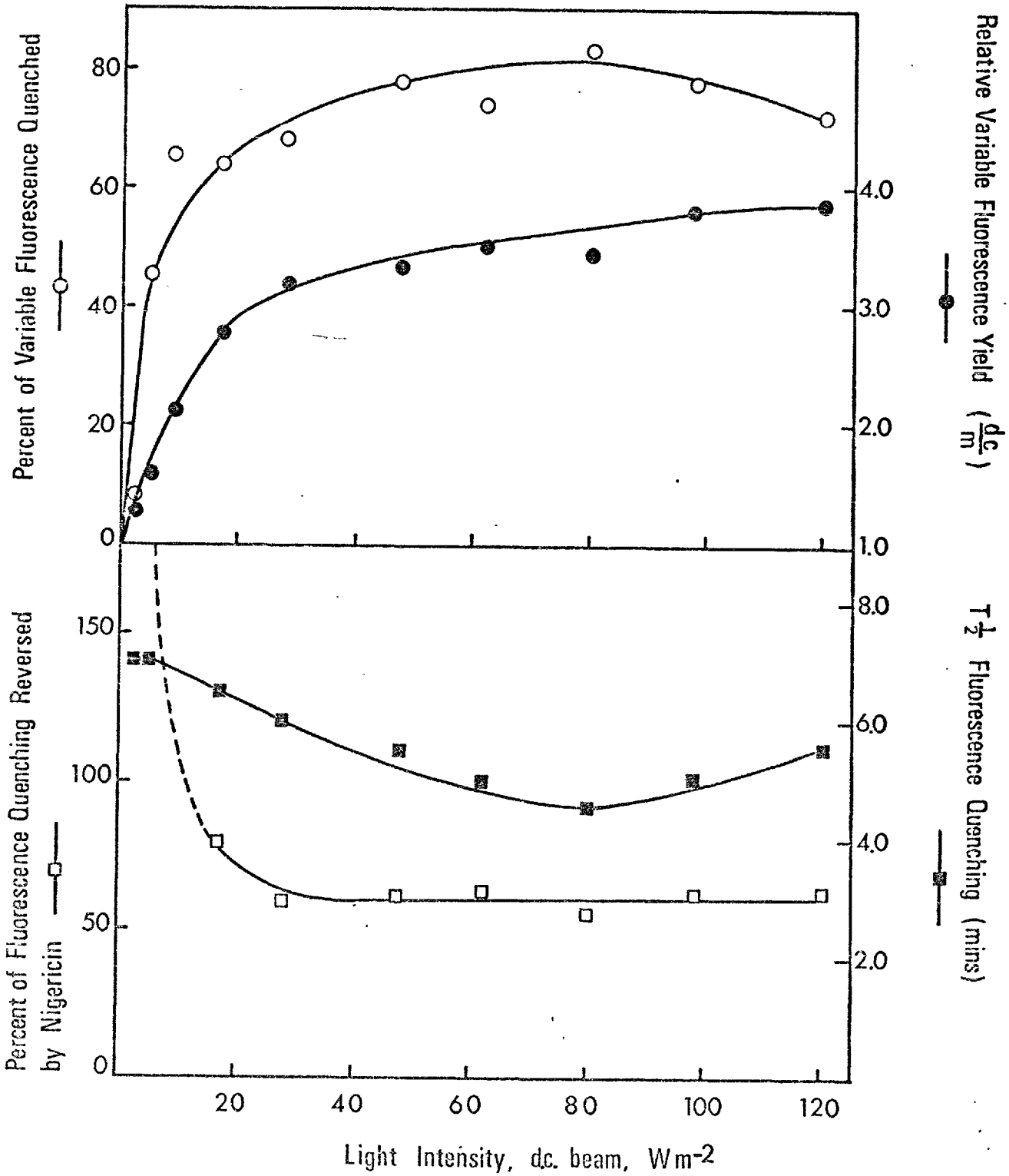


Fig. 3.3 Some effects of dc. light on modulated chlorophyll fluorescence excited by a weak modulated light: dependence on the intensity of dc. light. Experimental conditions as for Fig 3.2.

for dark adapted intact chloroplasts. The Q-dependent yield of variable fluorescence ($\frac{d.c.}{m}$) elicited by the d.c. actinic light increased with increasing d.c. light intensity, saturating at 80-100 Wm^{-2}. The extent of the subsequently observed slow fluorescence quenching also showed a similar light intensity dependence. This again indicates that slow fluorescence quenching only affects the variable portion of fluorescence yield. However, the magnitude of the steady state yield of variable fluorescence also reflects (though in a non-linear way) the rate of electron transport through PSII reaction centres. The correlation therefore between the yield of variable fluorescence initially seen and the extent of slow fluorescence quenching subsequently observed in d.c. illumination may also reflect the increased ability of chloroplasts at increasing light intensities to maintain greater transthylakoid pH gradients linked to non-cyclic electron transport. This is supported by the observed decrease in the half-time ($t_{\frac{1}{2}}$) required to attain the maximally quenched fluorescence state if light is raised to saturating intensity. A decreased$t_{\frac{1}{2}}$ might be expected from higher rates of non-cyclic electron transport and therefore of coupled proton pumping leading to more rapid development of the high energy state within the intact organelle. Figure 3.3(b) also shows the extent of fluorescence quenching which was reversed by addition of nigericin to the fully quenched state in d.c. illumination. At and above saturating light intensities, nigericin restored fluorescence to only 60% of the pre-quenched level. This contrasts to the 100% reversal of slow fluorescence quenching brought about by nigericin in the experiment shown in Figure 3.2. In fact, it was generally observed that the uncoupler sensitivity of slow fluorescence quenching observed in both intact chloroplasts and broken chloroplasts reconstituted with cations varied widely between chloroplast preparations. The reason for this is not clear, but like many other

photosynthetic properties (for example the ability to initially isolate intact chloroplasts in good yield) seems to be largely determined by the quality of spinach or other starting material used.

In the rest of the experiments reported in this thesis, only chloroplasts showing a high degree of uncoupler-sensitive slow fluorescence quenching were used for experimentation. Other workers[202] have studied slow fluorescence changes which were largely irreversible on darkening the suspension or addition of uncouplers and these results will be discussed in Section 3.3.

3.2.2. Studies with cation-specific ionophores on slow chlorophyll a fluorescence quenching in isolated chloroplasts

Barber and Telfer[194] concluded that the high fluorescing state which exists on illuminating dark-adapted isolated, intact chloroplasts was due to the presence of metal cations in the granal space. Initiation of light-induced electron transport and associated proton pumping caused these cations to be driven from the intrathylakoid space in exchange for incoming protons, and this caused the fluorescence lowering. In order to investigate the nature of these proposed cation fluxes, several cation-specific ionophores were used to study fluorescence changes in isolated chloroplasts under carefully controlled ionic conditions. Figure 3.4 shows typical time courses of such slow fluorescence changes in high actinic illumination (70-80 Wm^{-2}) in a medium free of inorganic cations (low salt buffer) other than 1 mM PGA added as the sodium salt. It can be seen that nigericin, which facilitates neutral exchange of K^+/H^+ across thylakoids[86,203] was ineffective at reversing fluorescence quenching under these conditions. When added together, CCCP and valinomycin, which increase the conductance of membranes to H^+ and K^+ respectively, also could not reverse fluorescence quenching. However,

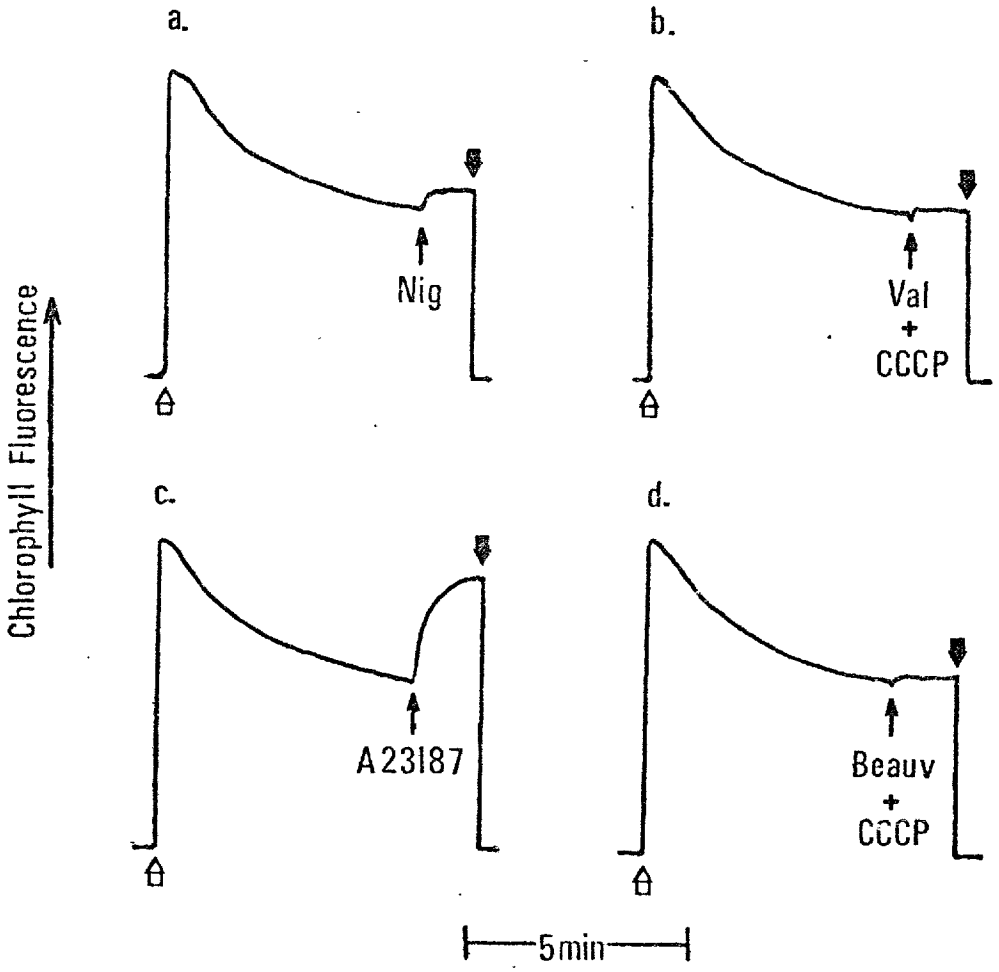


Fig. 3.4. Ability of various ionophore treatments to reverse light-induced, slow chlorophyll fluorescence quenching observed with intact chloroplasts suspended in low salt buffer containing 1mM PGA as the only added inorganic cations. Additions: (a) 0.05 µM nigericin; (b) 10^{-7} M valinomycic plus 2 x 10^{-7} M CCCP; (c) 2 µM A23187; (d) 0.3 µg ml^{-1} beauvaricin plus 2 x 10^{-7} M CCCP. Chloroplast concentration was 30 µg chlorophyll ml^{-1}. Other experimental conditions as for Fig 3.1.

ionophore A23187 which acts as a neutral exchanger of protons with divalent metal cations (Mg^{2+}, Ca^{2+}, Mn^{2-}, see Refs. 84,85) was able to restore quenched fluorescence to the high yield. When 2 mM K^+ was present in the external bathing medium (Figure 3.5), all three ionophore treatments successfully reversed fluorescence quenching of illuminated intact chloroplasts. At the low concentrations employed, CCCP plus valinomycin acted synergistically, showing the dual requirement for both K^+ and proton transport across thylakoids. Addition of 1 mM Mg^{2+} in place of 2 mM K^+ had little effect on the ability of nigericin, CCCP plus valinomycin or A23187 to bring about return of quenched fluorescence to the high yield.

These results appear to indicate that the endogenous stromal activity of divalent cations is sufficient to allow A23187 to dissipate the ΔpH gradient generated across the thylakoids of intact chloroplasts which is thought to bring about the fluorescence lowering. However, the inability of nigericin or CCCP plus valinomycin to reverse fluorescence quenching until 2 mM K^+ is added to the external medium suggests that the stromal activity of K^+ is rather low. In the absence of PGA, it was also found that monensin (facilitating Na^+/H^+ exchange) was also ineffective until 2 mM Na^+ was added to the reaction medium, indicating that stromal levels of this monovalent cation may also be low. The high apparant divalent/monovalent stromal activity suggests that either Ca^{2+} or Mg^{2+} (or possibly Mn^{2+}) may act as the main exchange cation for proton pumping in intact chloroplasts. Although A23187 shows approximately equal specificity for these divalent cations, the antibiotic beauvaricin has been reported[193] to discriminate between these cations in favour of Ca^{2+}. Beauvaricin also appears to transport Ca^{2+} electrophoretically across artificial bilayer membranes[204] and was therefore used together with the proton carrier CCCP to investigate whether Mg^{2+} or Ca^{2+} were involved in the fluorescence quenching process in intact chloroplasts. Figures 3.4

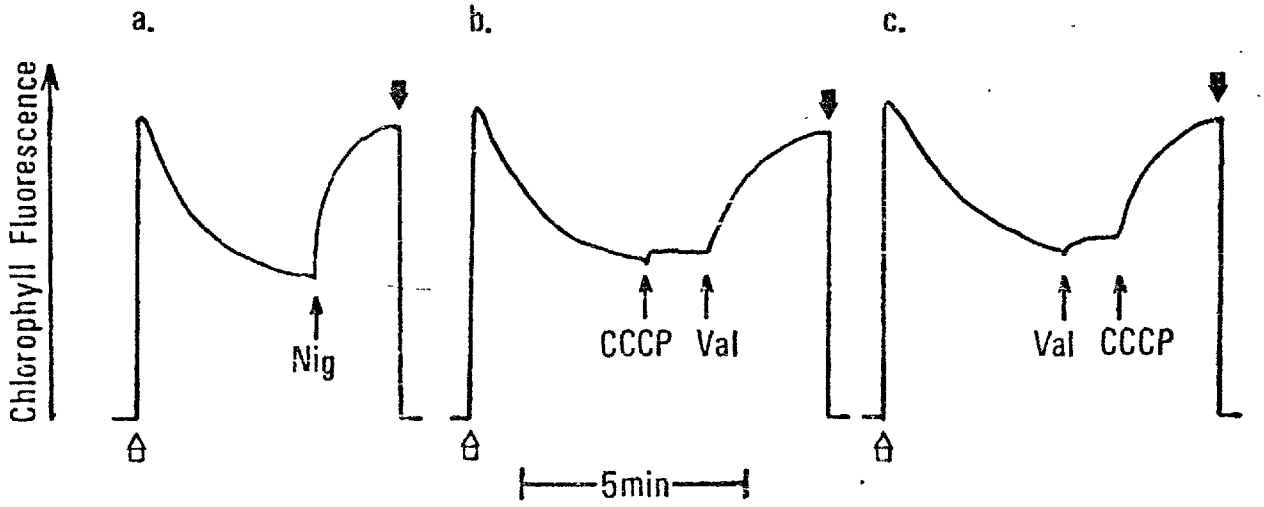


Fig. 3.5 Effect of 2mM KCl in the suspending medium on the ability of (a) 0.05 µM nigericin and (b), (c) 10^{-7} M valinomycin plus 2 x 10^{-7} M CCCP to reverse fluorescence quenching observed on illuminating intact chloroplasts suspended in low salt buffer. Other conditions as for Fig 3.4.

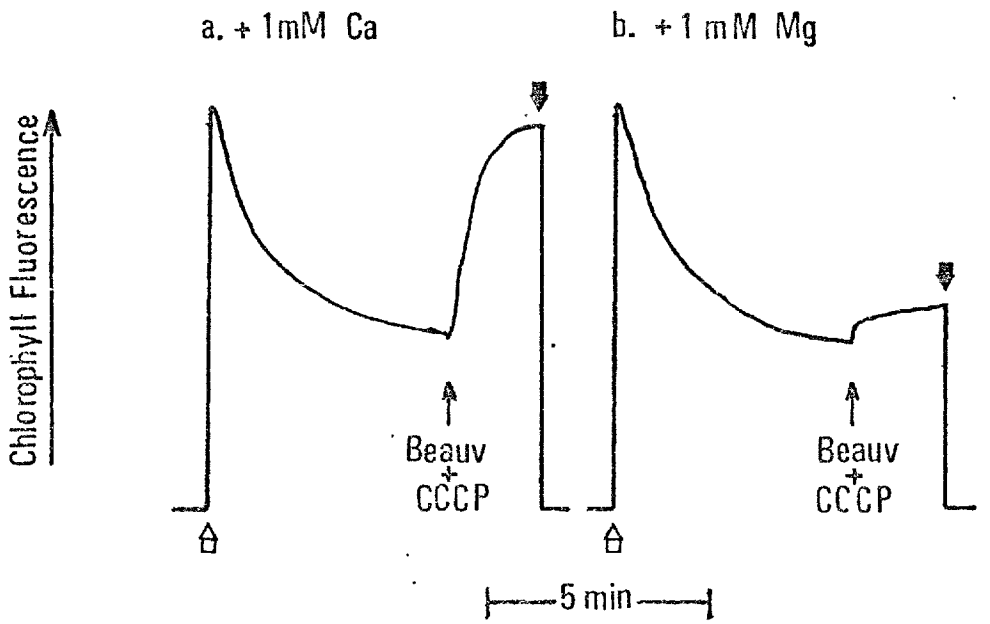


Fig. 3.6 Effect of (a) 1 mM $CaCl_2$ and (b) 1 mM $MgCl_2$ in the suspending medium on the ability of 0.3 µg beauvaricin plus 2 x 10^{-7} M CCCP to reverse fluorescence quenching observed on illuminating intact chloroplasts suspended in low salt buffer. Other conditions as for Fig 3.4.

and 3.5 show that beauvaricin plus CCCP were unable to reverse fluorescence quenching until 1 mM Ca^{2+} (but not 1 mM Mg^{2+}) was added to the medium. These results suggest that Mg^{2+} but not Ca^{2+} or monovalent cations is mainly involved in fluorescence quenching phenomena *in vivo*. Similar conclusions were reached by Telfer *et al*.[205] They found from ionophore studies of electron transport in isolated intact chloroplasts, that Mg^{2+} was the main exchange cation for proton uptake associated with generation of the ΔpH gradient of the transthylakoid high energy state.[205]

The apparent low activity of monovalent cations in the intact chloroplast was surprising in view of the high levels of K^+ and Na^+ previously reported to occur in this organelle.[11,104,105] The possibility that suspension of isolated intact chloroplasts in low salt media induced leakage of K^+ and Na^+ across the outer envelope was discounted by Telfer *et al*[205] from flame photometry analysis of the ionic levels within intact chloroplasts under these conditions. The full concentration curve for nigericin induced reversal of slow fluorescence quenching in intact chloroplasts in the absence of PGA confirms the finding that this antibiotic is ineffective even at very high concentrations when no external K^+ is present (Figure 3.7a). Similar results were obtained when OAA was added as electron acceptor. The effectiveness of high concentrations of nigericin in the presence of PGA (Figure 3.7b) was probably due to the sodium (1 mM) added with PGA. The results emphasise that under these conditions, the apparent stromal activity of chloroplast K^+ is low.

In contrast, ionophore A23187-induced reversal of fluorescence lowering in illuminated intact chloroplasts was little affected by the presence of 1 mM Mg^{2+} in the medium (Figure 3.8) except for a slight increase in the extent of reversal at all concentrations of this antibiotic.

From these results it may be concluded that Mg^{2+} is the main counterion to proton pumping in the intact organelle. However, it is necessary

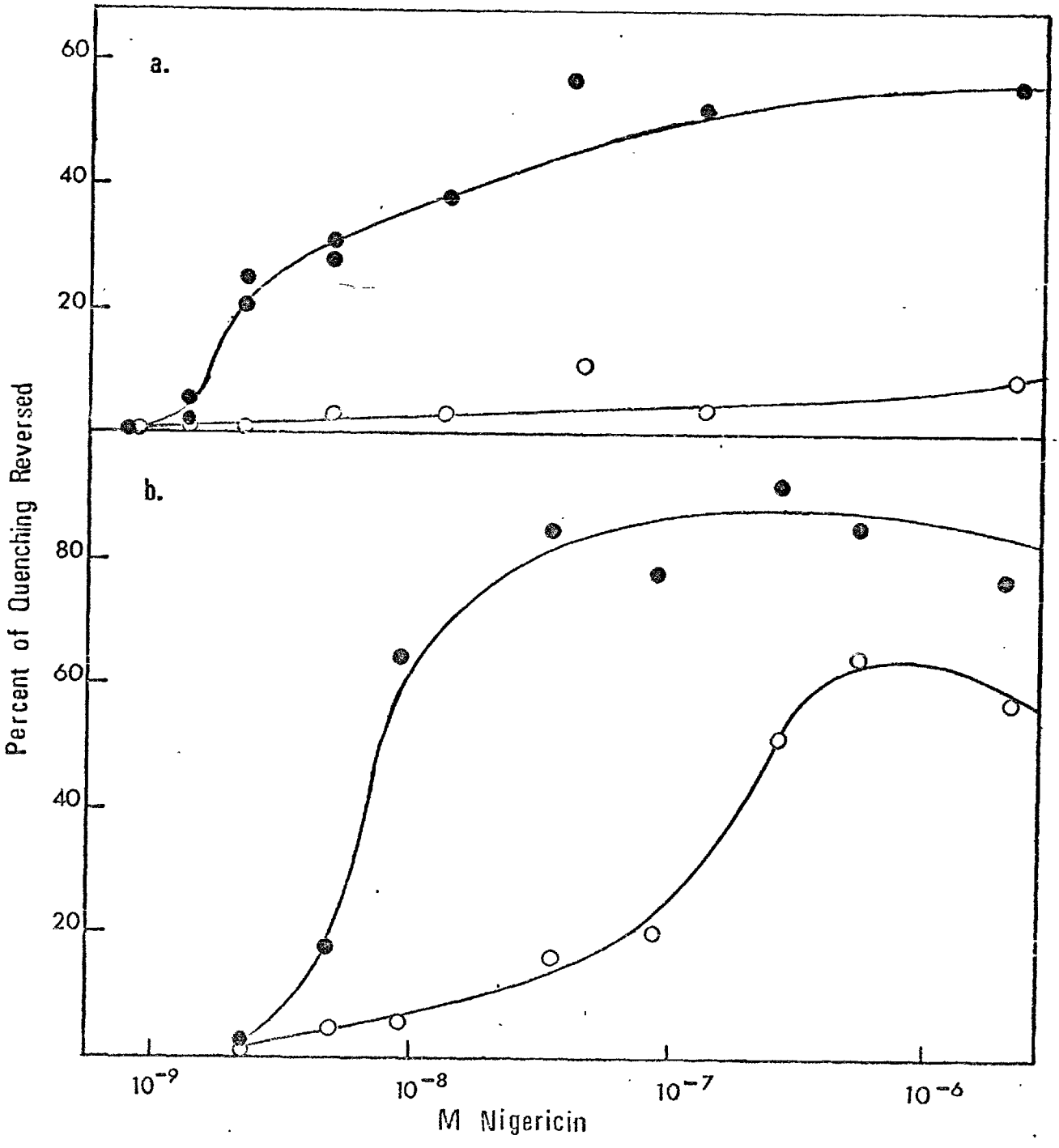


Fig. 3.7 The dependence on added K^+ of nigericin induced reversal of fluorescence quenching observed on illuminating isolated intact chloroplasts suspended in low salt buffer, (a) in the absence, and (b) in the presence of 1 mM PGA. Other conditions as in Fig 3.4. Denoting fluorescence levels as defined in Fig 3.1b, percent quenching reversed is given by (Fr - Fq)/(Fi - Fq) x 100. Open circles, in the absence of added K^+; solid circles, 2 min after adding 5 mM KCl to the same sample.

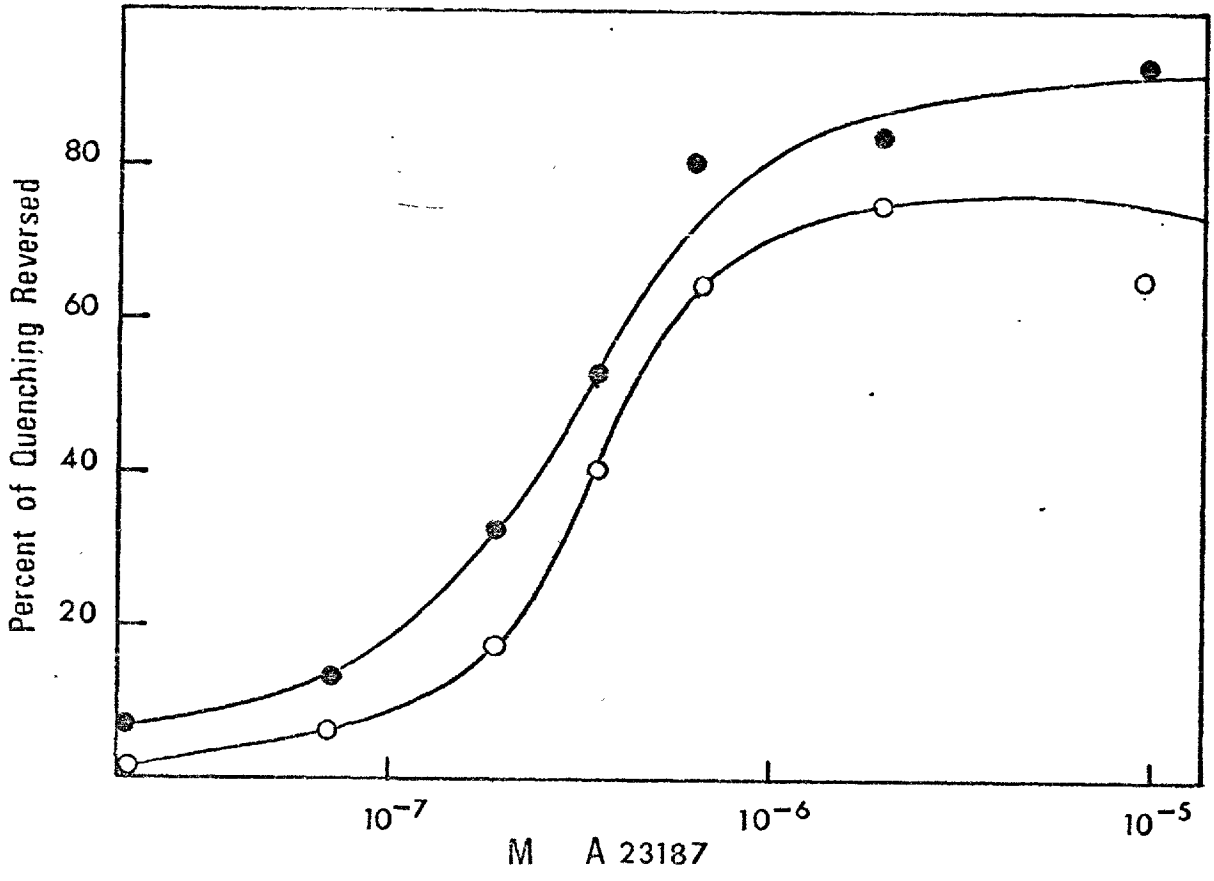


Fig. 3.8 The dependence on added Mg^{2+} of the ability of ionophore A23187 to reverse fluorescence quenching observed with illuminated intact chloroplasts suspended in low salt buffer. Open circles, in the absence of added Mg^{2+}; solid circles, 2 min after addition of 1 mM $MgCl_2$ to the same sample. Other conditions as for Fig 3.7b.

to assume that light-driven Mg^{2+}/H^{+} gradients are dissipated by A23187 but not nigericin, whereas K^{+}/H^{+} exchange would be promoted by nigericin but not A23187.

This assumption was tested by ionophore studies with osmotically broken chloroplasts reconstituted with 5 mM Mg^{2+} or 100 mM K^{+} so as to restore the initial high fluorescence on illumination and subsequent slow quenching of yield. In the presence of 5 mM $MgCl_2$, fluorescence quenching (which is presumably due to Mg^{2+}/H^{+} exchange across the thylakoid) was predictably reversed by ionophore A23187 but not nigericin until 5 mM K^{+} was also added to the medium (Table 3.1). These features are thus similar to ionophore sensitive fluorescence changes observed with intact organelles. However, on reconstituting the high energy state dependent fluorescence yield changes in broken chloroplasts with 100 mM K^{+}, the action of the two ionophores was quite different. Unlike intact organelles, Table 3.2 shows that both A23187 and nigericin were able to relieve the light induced quenched state. The effectiveness of A23187 was unexpected in this case as no divalent cations were added to the suspension medium. In order to study this effect more closely, the small and somewhat variable fluorescence quenching in cation reconstituted broken chloroplasts in the absence of added electron acceptors (see Tables 3.1 and 3.2) needed to be improved. It was found that a low concentration (5×10^{-8} M) of the electron acceptor methyl viologen (MeVi) (which is reoxidised by molecular oxygen) was sufficient to consistently increase the extent of quenching to 40% or more of the initial yield without causing too much overall oxidation of Q. This level of MeVi is well below the optimum for electron transport (10-100 μM) and presumably serves to stimulate sufficient coupled electron transport to create the maximum high energy state. Using this system, Figure 3.9 shows that A23187 reversal of fluorescence quenching in K^{+} reconstituted chloroplasts

TABLE 3.1.

Ionophore induced reversal of slow chlorophyll a fluorescence quenching reconstituted in broken chloroplasts[a] with 5 mM Mg^{2+}

Ionophore	Percent quenching[b]	Percent of quenching reversed[c]
2 μM A23187	28	69
0.1 μM Nigericin	26	14
0.1 μM Nigericin + 5 mM KCl	26	52

(a) Chloroplasts suspended in low salt buffer.

(b) Percent quenching defined as 100 $(F_i - F_q)/F_i$ using fluorescence levels denoted in Fig. 3.1b.

(c) Percent quenching reversed and other conditions as in Fig. 3.7.

TABLE 3.2.

Ionophore induced reversal of slow chlorophyll a fluorescence quenching reconstituted in broken chloroplasts with 100 mM KCl

Ionophore	Percent quenching[(a)]	Percent of quenching reversed[(a)]
2 μM A23187	20	65
0.1 μM Nigericin	7	140

(a) Defined and other conditions as in Table 3.1.

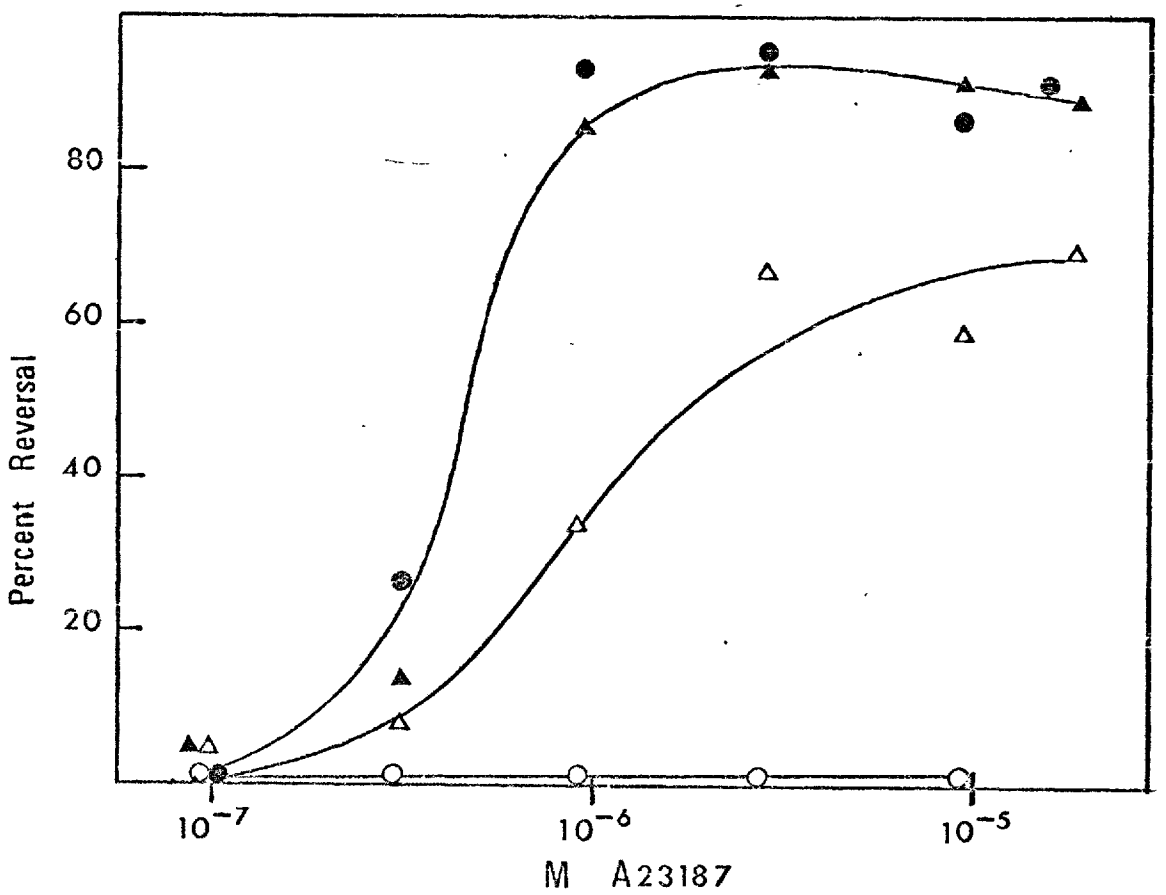


Fig. 3.9 Effect of Mg^{2+} and EDTA on the ability of ionophore A23187 to reverse chlorophyll fluorescence quenching observed on illuminating broken chloroplasts suspended in low salt buffer containing 100 mM KCl and 5 x 10^{-8} M MeVi. In this case, percent reversal is defined as the percentage of the control, which was the total reversal of fluorescence quenching obtained on adding saturating concentrations of A23187 and Mg^{2+} at the end of each sample run. Triangles, in the absence of EDTA; circles, in the presence of 0.5 mM EDTA; open symbols, in the absence of added Mg^{2+}; solid symbols, 2 min after adding 1 mM $MgCl_2$.

occurred in the absence of divalent cations and was only slightly improved on adding 1 mM $MgCl_2$ to the medium. However, when 0.5 mM EDTA was introduced into the cuvette, A23187 showed no ability at any concentration to reverse fluorescence quenching until 1 mM Mg^{2+} was also added. The relative effectiveness of A23187 in the absence of EDTA and $MgCl_2$ suggests that small amounts of Mg^{2+} are carried over from the isolation medium, or are released from the stroma on osmotically shocking intact chloroplasts. These low levels of Mg^{2+} (chaelatable with EDTA) are sufficient to enable A23187 to dissipate the ΔpH gradient by Mg^{2+} dependent uncoupling even in K^+ reconstituted broken chloroplasts. The results establish that under controlled conditions, K^+-dependent fluorescence changes are reversed only by nigericin and Mg^{2+}-dependent changes only by A23187. The possibility that results obtained with ionophore studies on fluorescence changes in intact chloroplasts might also have been influenced by residual amounts of divalent cations in the medium was then considered. Line 1 of Table 3.3 summarises the extent of fluorescence quenching observed in intact chloroplasts suspended in low salt buffer which was reversed by a period of darkness, addition of DCMU or uncoupling with A23187 or nigericin. In the case of nigericin, 5 mM KCl was also included in the reaction medium. DCMU fluorescence reversal was biphasic as previously reported.[194] The small, initial rapid phase which almost certainly represents the closing of PSII traps provided a convenient measure of the slow quenching which can be attributable to reoxidation of Q. This presumably occurs as electron transport attains maximal rates in the light. The remaining slow reversal of fluorescence brought about by DCMU was due to relief of high energy state quenching.

When EDTA was included in the reaction medium (Line 2, Table 3.3) dark, nigericin plus KCl, and DCMU induced reversal of fluorescence quenching of these intact chloroplasts were slightly inhibited. The

TABLE 3.3.

Inhibition by A23187 and EDTA of the reversibility of fluorescence quenching observed in intact chloroplasts

Addition	Percent of quenching reversed[a] by				
				DCMU	
	Dark	Nigericin[b] (0.1 μM)	A23187 (2 μM)	Fast phase	Fast and slow phase
None	60	83	63	15	77
0.5 mM EDTA[c]	43	79	16	18	64
0.5 mM EDTA[c] + 2 μM A23187	37	63	-	19	41

(a) Defined, and other conditions as in Table 3.1.

(b) Medium also contain 5 mM KCl.

(c) EDTA added before illumination. All other reagents were injected into the reaction mixture after a standard period of illumination which brought about fluorescence quenching.

effectiveness of A23187, however, was severely reduced. Line 3 of Table 3.3 shows that the quenched level of fluorescence not reversed by A23187 plus EDTA was relieved to a small extent by a subsequent period of darkness of addition of DCMU. Much of this DCMU reversal (50%) was observed as a fast phase and therefore represented closing of PSII traps. Nevertheless, in the absence of DCMU some reversal occurred in the dark with slow kinetics which could be requenched on a second period of illumination and returned to the dark level by nigericin plus KCl. Under these conditions (i.e. A23187 plus EDTA) and in the additional presence of KCl, nigericin was able to significantly reverse the quenched fluorescence level although again, this was less than the maximum reversal obtained in the absence of EDTA. The results show that although EDTA inhibits the ability of A23187 to reverse fluorescence quenching in intact chloroplasts, this effect is more complicated than that observed in broken chloroplasts. Not only does EDTA reduce the effectiveness of A23187, but also reduces the total reversibility of slow fluorescence quenching by any other method. These results cannot be explained simply on the apparant thermodynamic activities of cations in the stroma and medium but may include ionophore facilitated transport of cations across the chloroplast outer envelope. This point will be discussed further in Section 3.3. Overall, the results do indicate that the ionophore sensitivity of slow fluorescence yield changes in intact chloroplasts are more like those obtained on reconstituting broken chloroplasts with Mg^{2+} than K^{+}. This may be taken to indicate that Mg^{2+} fluxes contribute greatly to ionic fluxes in response to proton pumping in the intact organelle.

3.2.3. Effect of cations on slow quenching of chlorophyll a fluorescence supported by electron flow through PSI

The involvement of the ΔpH component of the high energy state in chlorophyll a fluoresence quenching can be observed in DCMU treated chloroplasts by stimulating electron flow through PSI which is coupled to proton pumping into the intrathylakoid space. Under these conditions, changes in fluorescence are not complicated by changes in the redox state of PSII associated with coupled electron flow through both photosystems.

As shown in Figure 3.10, low concentrations of DAD (25 μM), which stimulates cyclic electron transport around PSI, brings about fluorescence quenching in isolated, intact chloroplasts much like that seen before the sample was treated with the non-cyclic electron transport inhibitor DCMU. Such DAD induced quenching differed from 'normal', endogenous fluorescence lowering in the absence of DCMU and added cofactors in two respects. The dark reversal was notably faster ($t_{\frac{1}{2}}$ 15-30 seconds) than 'normal' quenching ($t_{\frac{1}{2}}$ 1.5-2 minutes) and often was not so extensive as seen in the same chloroplasts before addition of DCMU. The effect of ionophores on DAD catalysed fluorescence lowering, however, was identical to that observed with non-cyclic electron flow, and again it was necessary to add K^+ (5 mM) before nigericin was able to reverse fluorescence quenching of intact organelles suspended in low salt buffer.

The results suggest that DAD catalysed fluorescence lowering may also be due to removal of cations from their sites of action on development of the ΔpH gradient across the thylakoids. However, previous workers[160,161] found that DAD or phenazine methosulphate (PMS) brought about fluorescence quenching from the high or low fluorescence state of broken chloroplasts (treated with DCMU) in a manner which was essentially independent of the cation constitution of the medium. The concentration of DAD used[160] in these studies was 8-10 times that used in the experiment show in Figure 3.10.

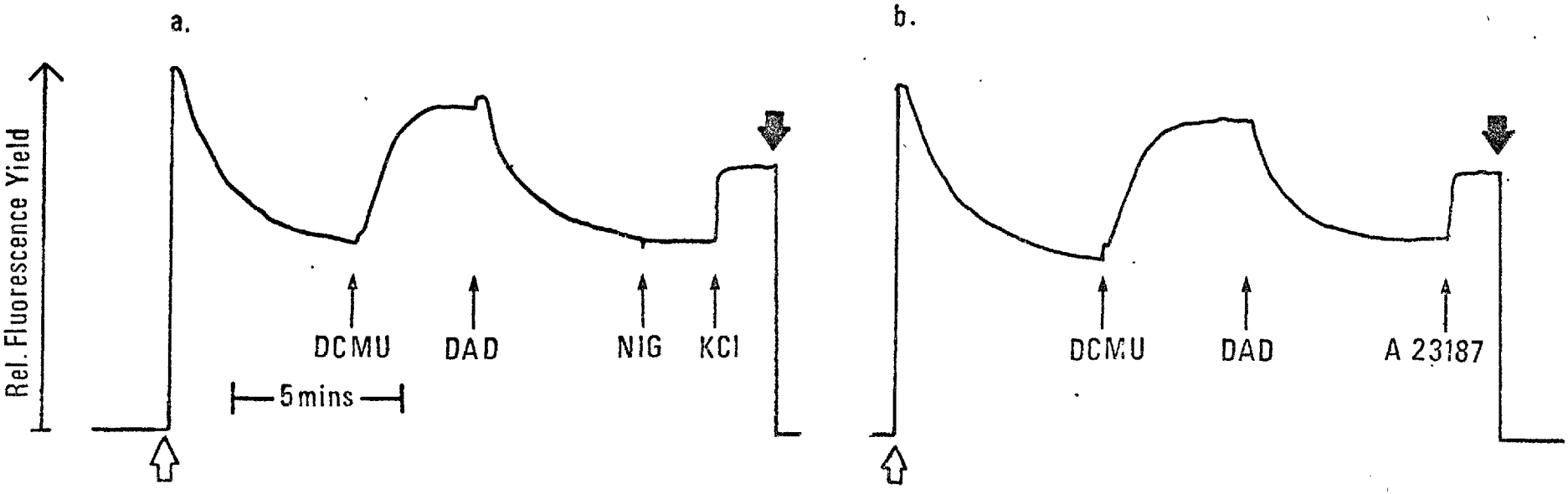


Fig. 3.10 Kinetics of chlorophyll fluorescence quenching induced by light-dependent non-cyclic electron flow (before addition of DCMU) and DAD stimulated cyclic electron flow (after addition of DCMU) in the same samples of intact chloroplasts. Suspending medium: low salt buffer contaiaing no electron acceptor; additions: 10 µM DCMU, 25 µM DAD and (a) 0.1 µM nigericin, 5 mM KCl, (b) 2 µM A23187

On reinvestigating DAD dependent fluorescence quenching observed in broken chloroplasts treated with DCMU, it was found that the extent, and cation sensitivity of the changes were strongly dependent on the concentration of cofactor used. Figure 3.11 shows that low concentrations (16.6 μM) of DAD induced significant fluorescence quenching only from the high fluorescence state of such chloroplasts pre-treated with 5 mM $MgCl_2$ (or 100 mM KCl). With only low levels of monovalent cations in the medium and fluorescence at the low level, this concentration of DAD brought about only slight fluorescence lowering. At higher levels of cofactor (250 μM), it was observed that DAD induced much more extensive fluorescence quenching from both the high and low fluorescence states which was largely independent of the presence or absence of 5 mM Mg^{2+} (Figure 3.11). At both high and low concentrations of DAD, fluorescence quenching showed the same sensitivity to uncouplers indicating the involvement of the high energy state in the fluorescence lowering process. Similar results were obtained when non-cyclic electron flow was induced through PSI only (in the presence of DCMU) using DAD or dichlorophenylindophenol (DCPIP) as electron donor (kept reduced with ascorbate) and adding MeVi as electron acceptor. Figure 3.12 shows that such slow quenching (with $DCPIPH_2$/MeVi couple) was very similar to that induced by non-cyclic electron through both PSII and PSI and did not significantly occur in the absence of cations which bring about the initial high fluorescing yield.

Figure 3.13 shows the extent of DAD stimulated fluorescence quenching (linked to cyclic electron flow) as a function of cofactor concentration. The double reciprocal plot has been reported by Wraight and Crofts[159] to be linear. As shown in Figure 3.13 the relationship between $1/\Delta F$ and 1/DAD was clearly biphasic. The point of transition between the two phases occurred between 10-20 μM DAD. The degree of biphasicity

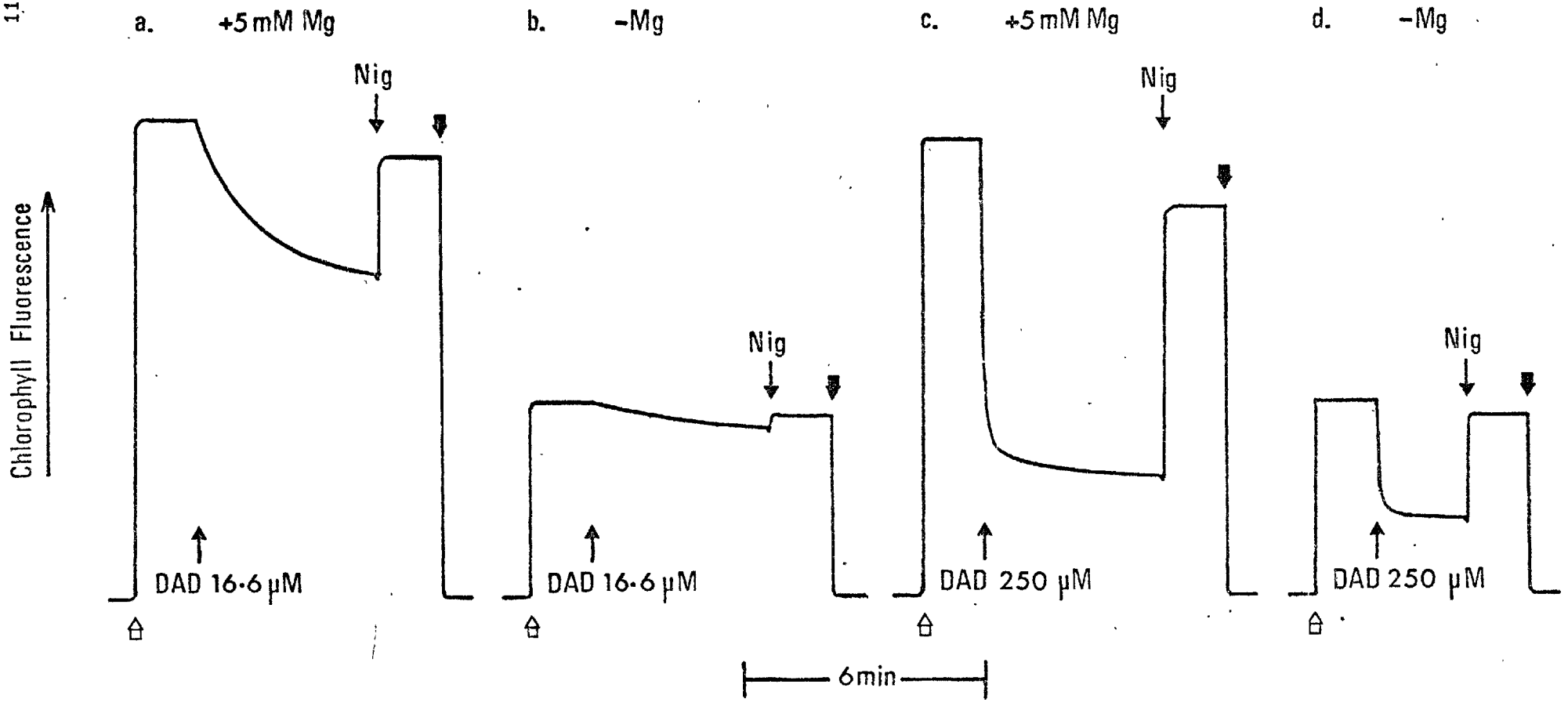


Fig. 3.11 Effect of 5 mM Mg^{2+} on the high energy state induced quenching of chlorophyll fluorescence supported by light dependent cyclic electron flow in the presence of low (16.6 µM) and high (250 µM) concentrations of the cofactor DAD. Broken chloroplasts were suspended in assay medium at 12 µg chlorophyll ml^{-1}, additions: 10 µM DCMU. Nigericin was added during illumination to a final concentration of 0.1 µM.

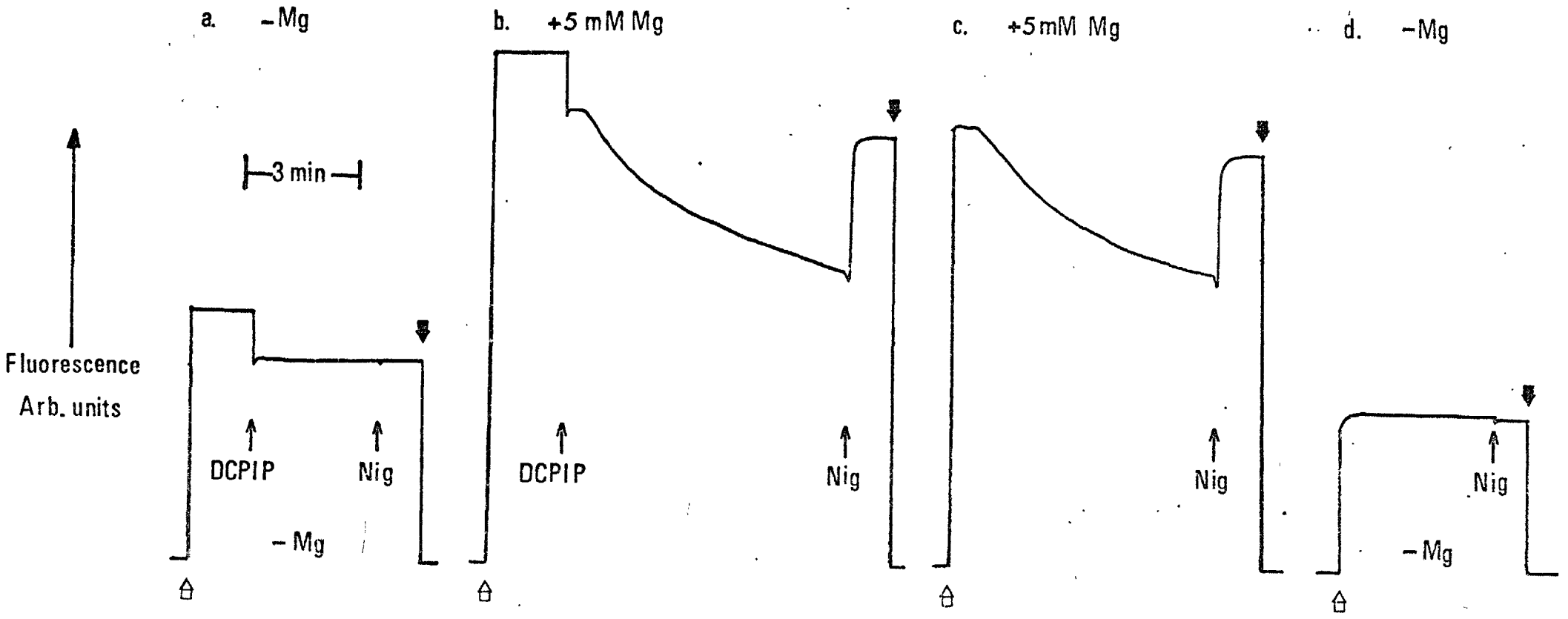


Fig. 3.12 Effect of 5 mM Mg^{2+} on high energy state induced chlorophyll fluorescence quenching supported by non-cyclic electron flow in illuminated broken chloroplasts: (a), (b) assay medium containing 10 µM MeVi, 0.5 mM Na azide, 2.5 mM Na ascorbate and 10 µM DCMU. Electron flow to MeVi mediated by PSI alone was initiated on addition of 100 µM DCPIP; (c), (d) endogenous non-cyclic electron flow through both PSII and PSI in broken chloroplasts suspended in assay medium in the absence of added electron acceptor. Other conditions as for Fig 3.1.

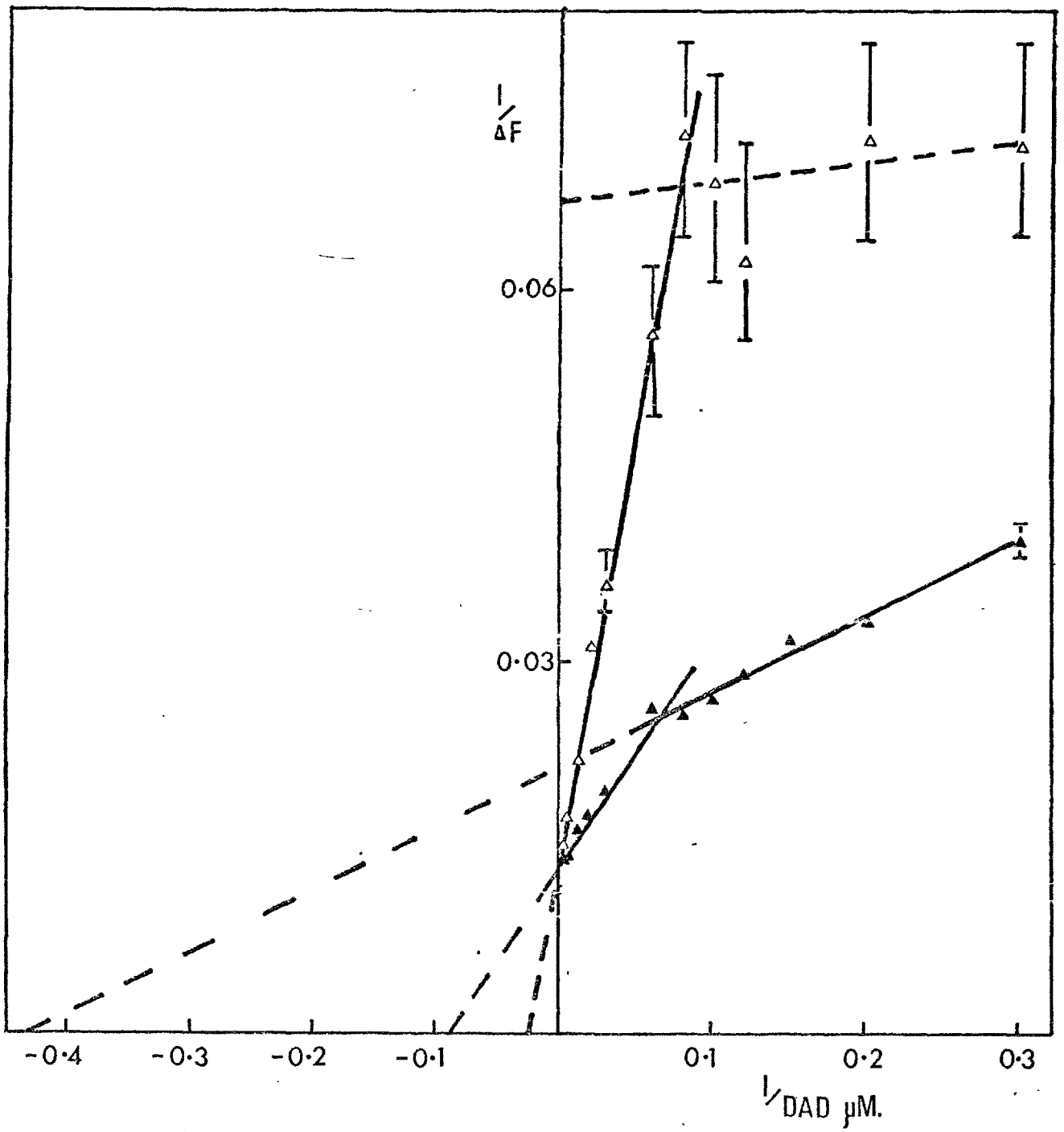


Fig. 3.13 Effect of 5 mM Mg^{2+} on the extent of high energy state induced fluorescence quenching supported by cyclic electron flow in the presence of DAD. Broken chloroplasts (12 μg chlorophyll ml^{-1}) were suspended in assay medium containing 10 μM DCMU in the absence (open triangles) or presence (solid triangles) of 5 mM $MgCl_2$. Using the notation of Fig 3.1, ΔF is defined as (Fi - Fq)/Fi, and the data is shown in the form of a double reciprocal plot. Note the biphasic nature of the plots both in the presence and absence of 5 mM Mg^{2+}.

varied somewhat between different chloroplast preparations but was most clearly seen in freshly isolated chloroplasts from healthy leaves which showed good fluorescence yield responses to cations. Occasionally, using aged chloroplasts or stock solutions of DAD several hours old, linear plots similar to those obtained by Wright and Crofts[159] were observed. The extent of DAD induced fluorescence quenching was also reduced in the latter preparations being similar to broken chloroplasts suspended only in monovalent cations at low concentrations (low fluorescing state). It seems likely that the failure of Wraight and Crofts to observe biphasic curves in double reciprocal plots may have been due to the poor response of their chloroplasts to cations.

The occurrence of two phases may suggest that DAD induces fluorescence quenching by two mechanisms. At concentrations of cofactor less than 20 μM (low Km phase). This phase appears to show an absolute requirement for cations at sufficient concentrations to bring about the high fluorescing state of chloroplasts. Omission of such cations (in this case 5 mM Mg^{2+}) severely reduced the extent of this low Km phase, which could further be reduced (essentially to zero) by reducing the level of monovalent cations in the medium from 17 mM to 5 mM. Fluorescence quenching over this range of DAD therefore resembles that induced by non-cyclic electron flow.

Above 20 μM, DAD induced fluorescence quenching occurred in the presence or absence of $MgCl_2$. However, $MgCl_2$ (5 mM) brought about an increase in the apparent Km of this phase from 12 μM to 40 μM, although these cations did not affect the maximum fluorescence (80%) quenched.

Reversal of fluorescence quenching by nigericin remained constant in extent (about 80% of fluorescence quenched) indicating that both the cation-sensitive and cation-insensitive fluorescence lowering was mainly dependent on the development of the transthylakoid pH gradient.

3.2.4. The relationship between chlorophyll fluorescence quenching and the extent of high energy state in broken chloroplasts

In order to further study the mechanism by which DAD at low and high concentrations influences chlorophyll fluorescence, and also to elucidate the role of metal cations in this process, the extent of the light induced thylakoid high energy state was estimated by two different methods.

The first of these methods involves the well known alkalisation of the suspending medium on initiating light induced coupled electron flow in broken chloroplasts. From the extent of the light/dark pH changes, the amount of protons taken up (into the intrathylakoid space) was quantitatively estimated. Table 3.4 shows the maximum extent of proton uptake observed in steady state illumination of broken chloroplasts suspended in a lightly buffered medium containing 10 mM KCl at pH 6.5. It can be seen that the extent of proton uptake under several conditions was not influenced by the presence or absence of 5 mM $MgCl_2$. Furthermore, DAD stimulated Δ pH changes in the presence of DCMU were essentially the same in total extent at both low (25 µM) and high (250 µM) concentrations of cofactor. The pH changes observed in the light were fully reversible on darkening the sample and were abolished in the presence of the uncoupler nigericin. In agreement with Krause,[196] the kinetics of proton uptake appeared to be consistent with those observed for slow chlorophyll fluorescence changes.

It must be noted, however, that the experimental conditions used for detection of pH changes in illuminated broken chloroplasts were different to those under which fluorescence measurements were made. For pH measurements, chloroplasts were illuminated with red light to avoid artifactual responses of the pH electrode observed under blue illumination. Also, a lower pH of 6.5 (c.f. pH 7.6 for fluorescence measurements) was employed to minimise drift to a more acid pH in such lightly buffered media.

TABLE 3.4

Extent of proton uptake from the external medium observed on illuminating broken chloroplasts[a]

Addition	$MgCl_2$ (5 mM)	H^+ uptake (μ equivalents/mg chlorophyll)
None[b]	-	0.19 ± 0.04
None[b]	+	0.16
3.3×10^{-5} M DCMU	±	0.00
DCMU + 25 μM DAD	-	0.24
DCMU + 25 μM DAD	+	0.26
DCMU + 250 μM DAD	-	0.28
DCMU + 250 μM DAD	+	0.28

(a) Broken chloroplasts (38 μg chlorophyll ml^{-1}) were suspended in 0.33 M sorbitol, 20 mM KCl, 10^{-5} M DCMU, 1 mM HEPES pH 6.5 (see Section 2.6).

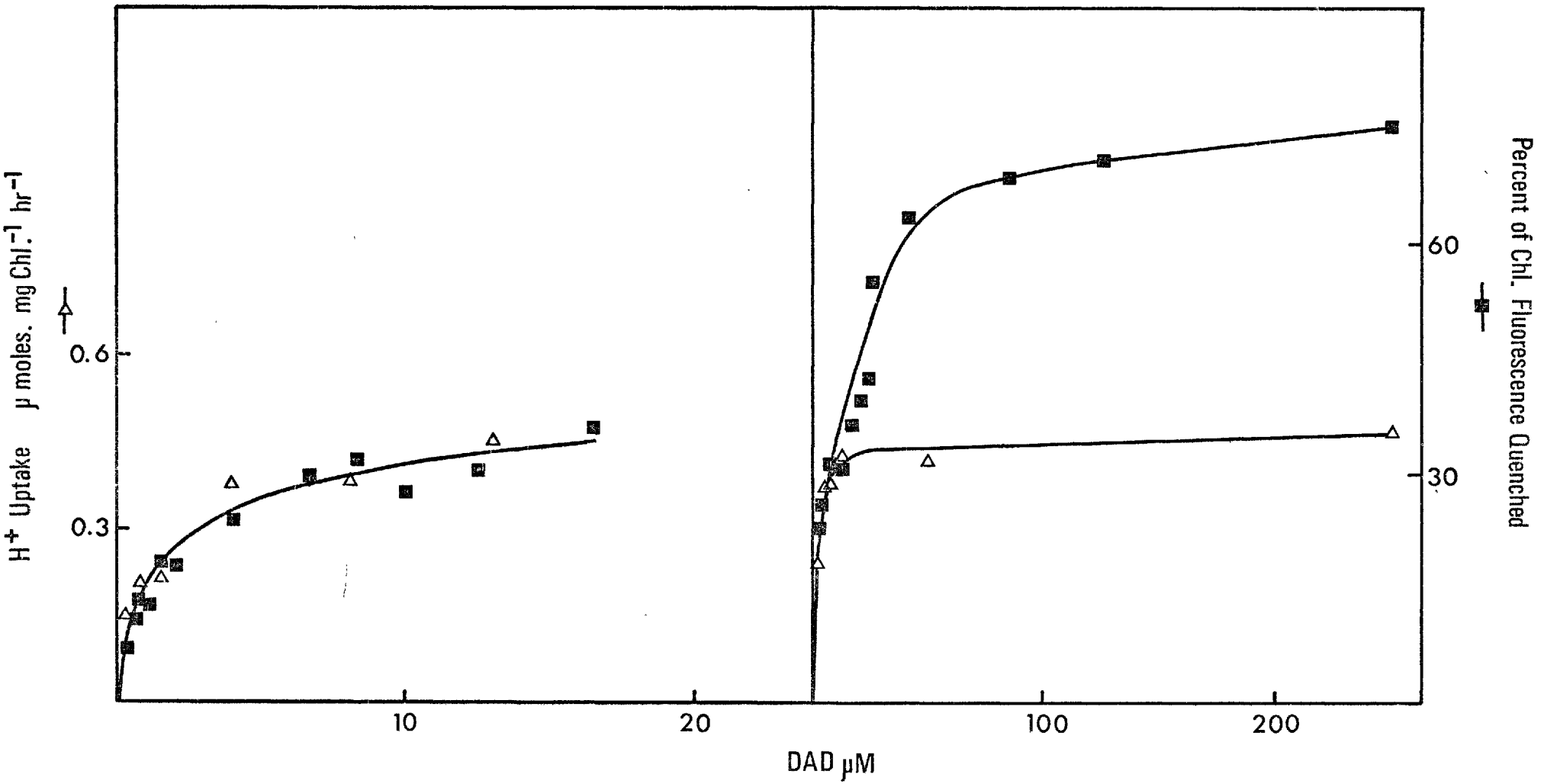
(b) DCMU omitted from medium.

However, the intensity of red light was the same (70-80 Wm^{-2}) as that used for fluorescence measurements, and essentially identical results (but with more experimental uncertainty) were observed in media of pH 7.5.

Bearing these points in mind, the results show that the presence or absence of 5 mM Mg^{2+} in the medium does not affect the extent of the ΔpH gradient maintained by broken chloroplasts in the light. The fact that slow fluorescence quenching induced by low concentrations of DAD (or non-cyclic electron flow) is only seen in the presence of these cations cannot be due to increased ΔpH gradients of thylakoids under these conditions. Furthermore, cation insensitive fluorescence quenching observed at higher concentrations of DAD was not correlated with an increased ΔpH gradient. This result is shown in more detail in Figure 3.14 which shows the concentration curve for DAD stimulated proton uptake observed with DCMU treated broken chloroplasts in the presence of 5 mM Mg^{2+}. Also plotted in this figure for comparison is the fluorescence quenching induced by DAD taken from another experiment. Increase in the extent of proton uptake correlates well with the extent of fluorescence quenching observed with DAD concentrations up to approximately 12 μM (i.e. with the low Km phase of Figure 3.13). Above 12 μM DAD, the extent of cofactor induced fluorescence quenching in the light continued to increase with DAD concentration, but there was little or no corresponding increase in H^{+} uptake from the medium.

Another method of measuring the extent of light induced high energy state of thylakoids is the uptake and accumulation of amines of high pK (> 9) into the intrathylakoid space which occurs in response to any ΔpH gradient existent across the membrane. According to the modelof Schuldiner *et al*[206] this results in a decrease in the fluorescence yield of fluorescent amines such as 9-amino acridine (9-AA), from which the extent of the ΔpH gradient can be quantitatively estimated. Others, however,[207,208]

Fig 3.14 Comparison of the extent of proton uptake from the suspending medium and chlorophyll fluorescence quenching observed in steady-state illumination of broken chloroplasts in the prescence of 10 μM DCMU, 5 mM MgCl and DAD. For H^+ uptake, broken chloroplasts were suspended in a lightly buffered medium of pH 6.5(other conditions are given in Section 2.6). For fluorescence measurements, conditions were the same as described in Fig 3.13.


H+ Uptake μ moles. mg Chl.-1 hr-1
0.6
0.3
10
20
100
200
DAD μM
60
30
Percent of Chl. Fluorescence Quenched

have cast doubt on the quantitative aspects of this model and have concluded that 9-AA fluorescence quenching is also caused by changes in the binding of this fluorescent probe to the thylakoid. In this latter model 9-AA fluorescence lowering is due to membrane conformational changes in response to the generation of the light induced high energy state.

Despite the disagreement as to the exact mechanism by which 9-AA fluorescence quenching occurs there seems no doubt that these changes in some way monitor events associated with generation of the chloroplast high energy state. For this reason 9-AA fluorescence changes were studied simultaneously with chlorophyll fluorescence quenching. As shown in Figure 3.15, PSI dependent cyclic or non-cyclic flow supported by DAD in DCMU treated broken chloroplasts brought about nigericin reversible quenching of 9-AA fluorescence. The extent of 9-AA fluorescence quenching saturated at much lower DAD concentrations than did chlorophyll fluorescence lowering. Like proton uptake, 9-AA fluorescence quenching showed little dependency on Mg^{2+} in the medium. Chlorophyll fluorescence changes, however, showed the usual requirement for cations particularly at low DAD concentrations. The results of 9-AA fluorescence and proton uptake studies suggest that the low Km, cation dependent phase of DAD induced chlorophyll fluorescence quenching is directly related to the extent of the high energy state developed by the thylakoids. This type of chlorophyll fluorescence change seems to correspond to the 'physiological' quenching observed in intact and broken chloroplasts in the absence of artificial cofactors.

The mechanism by which this 'physiological' fluorescence quenching occurs is not established. As Figure 3.16 shows, the ability of cations (in this case 5 mM Mg^{2+}) to induce the high fluorescing state of DCMU treated chloroplasts is dependent on the external pH of the suspending medium. Below pH 6.0, this cation sensitive fluorescence increase was

Fig 3.15 Comparison of the extent of high energy state induced quenching of 9-AA and chlorophyll fluorescence yields observed in steady-state illumination of broken chloroplasts suspended in low salt buffer in the presence of DAD. (a) Non-cyclic electron flow through PSI in the presence of 100 µM MeVi, 0.5 mM Na azide and 5 mM Na ascorbate; (b) cyclic electron flow . Chloroplast concentration was 12 µg chlorophyll ml^{-1}, and all samples contained 10 µM DCMU. Symbols: open, 9-AA fluorescence; closed, chlorophyll fluorescence; squares, inthe absence of added Mg^{2+}; circles, in the presence of 5 mM $MgCl_2$.

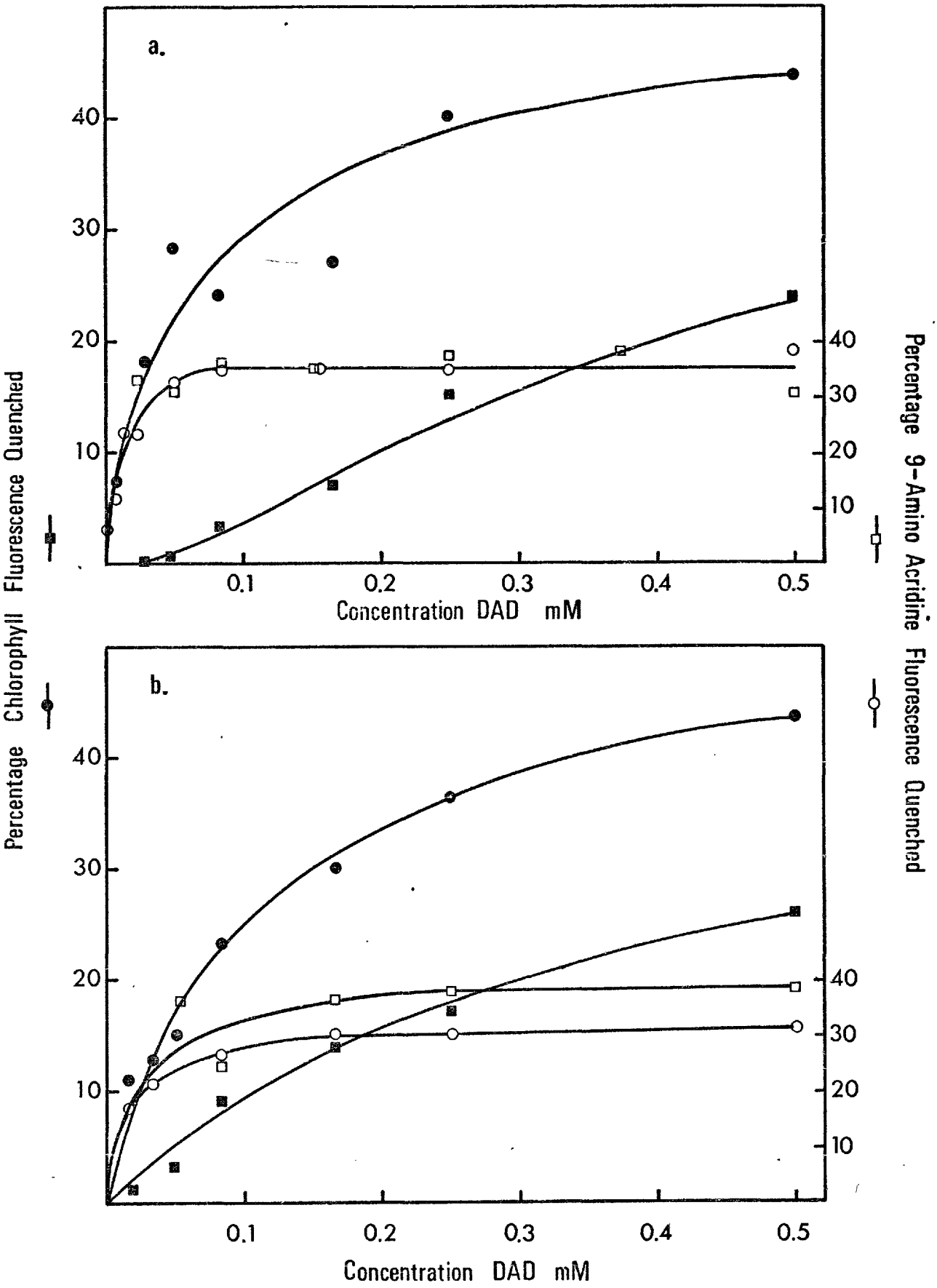
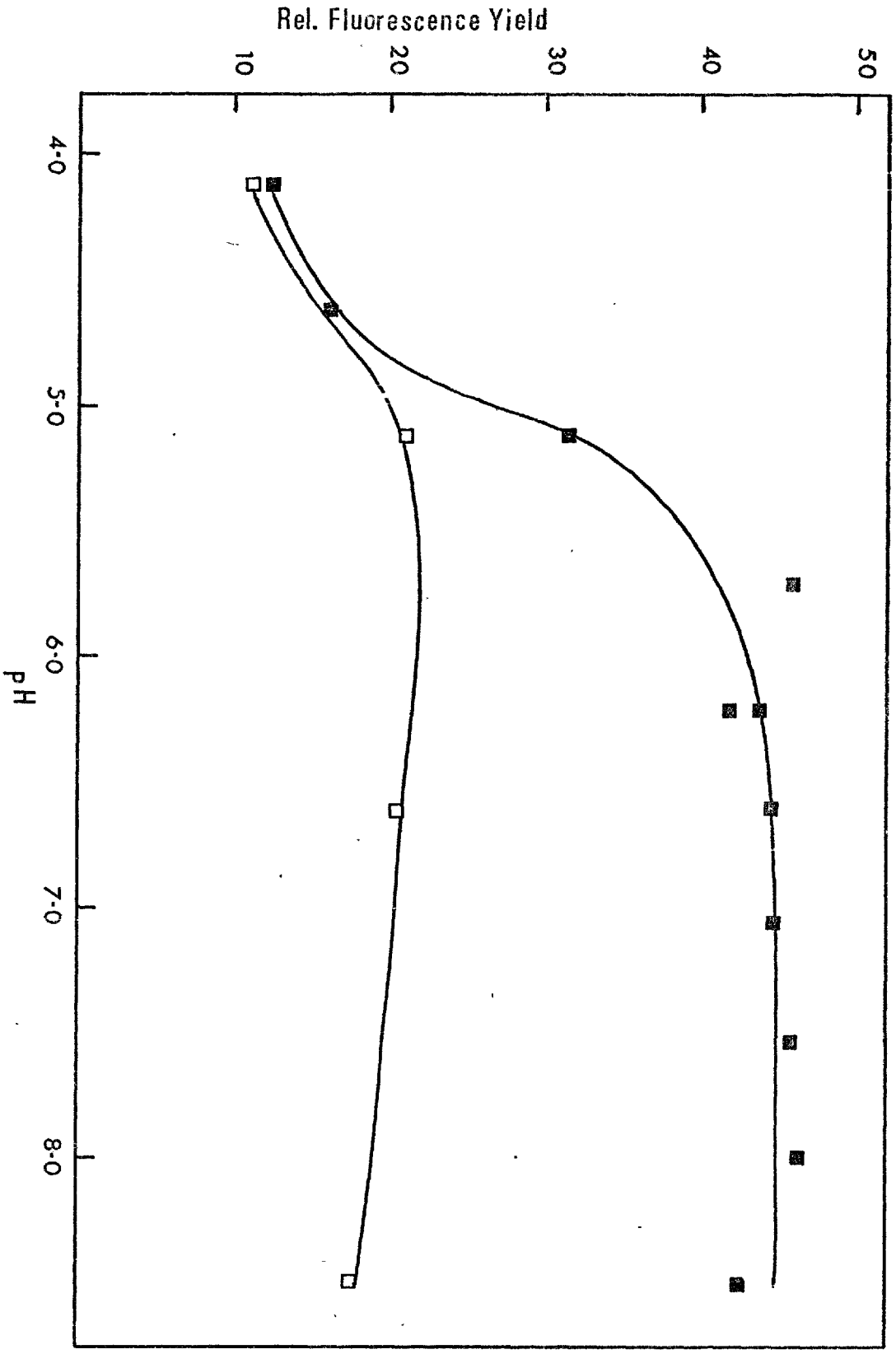

a.
b.
40
30
20
10
0.1
0.2
0.3
0.4
0.5
Concentration DAD mM
Percentage Chlorophyll Fluorescence Quenched
Percentage 9-Amino Acridine Fluorescence Quenched

Fig. 3.16 pH dependence of the Mg^{2+} stimulated increase in fluorescence of broken chloroplasts. For measurements below pH 6.0, broken chloroplasts (20 μg ml^{-1}) were suspended in 0.33M sorbitol containing 10 μM DCMU, 5 mM succinic acid, 5 mM HEPES, brought to pH with tris base. For measurements above pH 6.0, succinic acid was replaced by 5 mM HEPES. This did not significantly affect fluorescence levels at pH 6.0. All samples were preincubated at the appropriate pH for 10 minutes; open squares, in the absence of added Mg^{2+}; solid squares, plus 10 mM $MgCl_2$.


Rel. Fluorescence Yield
10
20
30
40
50
pH
4·0
5·0
6·0
7·0
8·0

progressively abolished and completely suppressed at pH 4.5. This result is therefore consistent with the hypothesis that light-induced fluorescence lowering is due to displacement of cations from their effective sites of action on the inner side of the thylakoid membrane as the internal pH of the thylakoid space is lowered by proton pumping. If such fluorescence changes are due to competition between protons and cations for fixed negative charges on the membrane it might be expected that increasing the external concentration of cations in medium would inhibit fluorescence quenching. However, increasing Mg^{2+} concentration from 3 to 30 mM did not inhibit, but rather increased the extent of fluorescence quenching induced by a very low concentration of DAD (4 μM, see Table 3.5). This result may indicate that fluorescence quenching does not depend on cation displacement, but rather the protonation of the inner side of the thylakoid as discussed in Section 3.3.

At high concentrations of DAD (> 20 μM) fluorescence quenching is clearly not solely dependent on cations. The extent of fluorescence quenching increases with increasing DAD concentrations, whereas the extent of the high energy state is maximal and constant. This type of fluorescence quenching is not seen in the absence of artificial co-factors and may therefore depend on some non-physiological process. The possibility was investigated that DAD may interact directly with the excited chlorophylls in the bulk pigment bed in the thylakoid to quench fluorescence. In fact, DAD induced irreversible quenching of chlorophyll fluorescence in chloroplasts treated with DCMU and nigericin plus KCl to eliminate high energy state effects was observed to occur under certain conditions. As shown in Table 3.6 high concentrations of DAD (1 mM) chemically oxidised with excess ferricyanide considerably decreased the fluorescence of these DCMU poisoned, uncoupled chloroplasts in media of pH 4.5. Oxidised DAD did not quench fluorescence under similar conditions

TABLE 3.5.

Effect of Mg^{2+} on light induced chlorophyll fluorescence quenching in broken chloroplasts[(a)] in the presence of a limiting concentration[(b)] of DAD.

Mg^{2+} (mM)	Extent of DAD induced fluorescence quenching (percent)
3	20
15	23
30	28

(a) Chloroplasts suspended in assay medium containing 10^{-5} M DCMU.

(b) 4 µM DAD.

TABLE 3.6.

Effect of oxidised DAD[a] on chlorophyll fluorescence at low and high pH.

pH	Additions	Fluorescence (arb. units)	
		Broken chloroplasts	Acetone extracted chlorophyll[d]
7.6[b]	None	100	100
7.6	1 mM DAD	93	100
4.5[c]	None	90	100
4.5	1 mM DAD	17	60

(a) All media contained 2 mM FeCy to completely oxidise DAD.

(b) Low salt buffer containing 10 mM KCl, 0.1 µM nigericin and 10^{-5} M DCMU.

(c) 0.33 M sorbitol, 5 mM HEPES, 5 mM succinic acid, 10 mM KCl, 0.1 µM nigericin, 10^{-5} M DCMU.

(d) Extracted from broken chloroplasts with 80% acetone and resuspended in either of above media to give final acetone concentration of 40%.

in media of pH 7.6. Similar results were obtained with a membrane-free solution of chlorophyll extracted from thylakoids with acetone and re-dissolved in buffered 40% acetone. In this case, however, fluorescence quenching by oxidised DAD was less marked at pH 4.5 than that observed with unextracted chloroplasts. The ascorbate reduced form of DAD at concentrations of 1 mM did not decrease fluorescence intensity of uncoupled chloroplasts or chlorophyll in acetone at either pH 4.5 or pH 7.6.

Measurement of the ferricyanide oxidised absorption spectrum of DAD at pH 4.5 revealed the presence of a sharp band at 478 nm (ε_{max} 1.35 ($\pm$0.2 mM^{-1} cm^{-1}) together with a weak broad absorption band centred around 690 nm. These absorptions bands could not be detected in oxidised DAD solutions maintained at pH 7.6 nor in solutions of the ascorbate reduced form of DAD at either pH 4.5 or pH 7.6. The apparent quenching of fluorescence in uncoupled chloroplasts or chlorophyll in acetone induced by oxidised DAD at pH 4.5 may therefore be due to absorption of emitted fluorescence and incident light ('inner filter effect'). Comparison of acetone extracted chlorophyll with poisoned broken chloroplasts revealed that DAD-induced fluorescence lowering at pH 4.5 of the former may be due entirely to the 'inner filter effect'. However, the much greater fluorescence quenching of uncoupled chloroplasts by oxidised DAD under similar conditions cannot be accounted for by 'inner filter effects'. It appears that the oxidised cofactor must also interact with chlorophyll <u>in the membrane</u> to directly quench excited state chlorophyll by other mechanisms. Using uncoupled chloroplasts this latter process apparently occurs at pH 4.5 but not at pH 7.6. At pH 7.6 cation insensitive fluorescence quenching induced by DAD is only seen on illuminating coupled chloroplasts and is abolished by uncouplers such as nigericin. It seems therefore that this type of quenching requires the presence of

the chloroplast high energy state which is known to lower the pH of the intrathylakoid space to pH 4-5.[78,79] As discussed in Section 3.3, cation insensitive DAD-induced fluorescence quenching may therefore be due to the accumulation of the oxidised cofactor in the intrathylakoid space. On generating the high energy state in illuminated chloroplasts, the lowered internal pH may create conditions conducive to DAD quenching of fluorescence by direct mechanisms.

3.2.5. Low temperature emission spectra

Results presented in the previous section indicated that DAD-induced fluorescence quenching depends on the generation of the chloroplast high energy state. The mechanism by which DAD-induced fluorescence quenching occurs seems to be different when low and high concentrations of cofactor are employed. Analysis of fluorescence emission spectra at 77°K of chloroplasts at the high initial, and at the DAD dependent light induced quenched state supports this contention (Table 3.7). At 77°K, fluorescence at 685 nm and 695 nm is thought to originate from PSII and emission at 730 nm from PSI. The ratio of PSII fluorescence to PSI emission is given as $F\frac{685}{730}$ and $F\frac{695}{730}$. It can be seen that addition of $MgCl_2$ (or 100 mM KCl) to dark pretreated chloroplasts considerably increases these low temperature emission ratios. This has been taken to indicate that cations inhibit spillover of excitation energy from PSII to PSI and thereby increase PSII fluorescence at low temperature at the expense of PSI. If DAD-induced fluorescence quenching (at low concentrations) reverses this process (promotes spillover), it would be expected that these low temperature emission ratios would be decreased. As shown in Table 3.7, this was only observed to occur in chloroplasts preilluminated in the presence of high concentrations of cations and low levels of DAD. In the absence of $MgCl_2$, low levels of DAD brought about a small increase

TABLE 3.7.

Ratio of PSII (F685, F695) to PSI (F730) fluorescence emission from broken chloroplasts at liquid nitrogen temperature ($77^{o}K$) after different pre-treatments at room temperature[(a)]

Additions		Preillumination	Emmission ratio at $77^{o}K$	
DAD (μM)	Mg^{2+} (5 mM)	(D = Dark) (L = Light)	$F\frac{685}{730}$	$F\frac{695}{730}$
20	+	D	1.51 ± 0.05	1.64 ± 0.05
20	+	L	1.45	1.58
20	-	D	0.93	0.91
20	-	L	0.98	0.91
250	+	D	1.48	1.66
250	+	L	2.77	2.25
250	-	D	1.04	1.00
250	-	L	2.00	1.40

(a) For other details see Section 2.2.4.

in the $F\frac{685}{730}$ ratio. At high cofactor concentrations, large increases in the $F\frac{685}{730}$ and $F\frac{695}{730}$ ratios were observed, and large scale quenching of the overall fluorescence yield at low temperature also occurred. These results support the idea that DAD at high concentrations quenches chlorophyll excited states in a manner which is enhanced when a ΔpH gradient exists across the thylakoids. The observed peak emission ratios at 77°K indicate that such quenching was relatively more efficient for chlorophyll of PSI than of PSII (and more efficient for chlorophyll emitting at 695 nm than 685 nm within PSII) at these temperatures.

Apparently, the DAD/high energy state quenching of chloroplast fluorescence pre-established at room temperature is more efficient on freezing the samples to liquid nitrogen temperatures. This can be seen using low concentrations of DAD, which did not significantly induce quenching from the low yield at room temperature when chloroplasts were suspended in media which contained 10 mM KCl. However, at low temperatures, low concentrations of DAD did increase the $F\frac{685}{730}$ ratio of preilluminated samples under these conditions. When 5 mM Mg^{2+} was included in the medium, a decrease in this ratio occurred. These results may indicate that in the presence of 5 mM Mg^{2+}, low levels of DAD bring about fluorescence quenching which involves an increase in spillover of excitation energy from PSII to PSI. However, the spillover changes appear to be obscurred by cation insensitive fluorescence quenching which tends to increase the $F\frac{685}{730}$ ratio.

3.2.6. Effect of metal cations on enhancement phenomena in broken chloroplasts

In the absence of non-physiological cofactors such as DAD, slow fluorescence quenching was observed in broken chloroplasts only in the presence of divalent (5 mM) or monovalent (100 mM) cations in the suspending

medium. These levels of cations are similar to those required to create the initial high fluorescing state by a mechanism proposed to involve a decrease in spillover of absorbed excitation energy from PSII to PSI.[157,169,170] It seems likely, therefore, that the slow, high energy state dependent fluorescence quenching from the high initial yield may involve a reversal of the cation dependent inhibition of spillover. Such antagonistic effects of cations and high energy state on the distribution of excitation energy between PSII and PSI may play a role in the mechanism controlling state I/II changes observed *in vivo*.[155]

Before exploring this possibility, it was deemed necessary to investigate whether such cation induced changes in fluorescence (in the absence of the high energy state) do reflect changes in spillover as suggested by most workers[97,128,157,172,174,177,194] but contested by others.[171,209]

The antagonistic effects of PSI and PSII lights on the Q-dependent fluorescence yield of chloroplasts[136] provides a useful method of measuring the relative distribution of excitation energy between PSII and PSI under any particular set of experimental conditions. Figure 3.17 shows such effects on broken chloroplasts uncoupled with nigericin and KCl to eliminate high energy state effects on chlorophyll fluorescence. When intially suspended in low salt buffer and therefore in the low fluorescing state, addition of d.c. PSI light had little effect on the fluorescence induced by a weak, modulated blue light (used here as PSII light). However, after adding 5 mM $MgCl_2$ (or 100 mM KCl, not shown), the overall increase in fluorescence induced by these cations was accompanied by an increased ability of PSI light to quench fluorescence.

It seems that in the presence of these levels of cations (5 mM divalent or 100 mM monovalent), the blue light overexcites PSII relative to PSI. This causes an overall partial reduction of Q to occur in weak, blue illumination. Addition of a second PSI light then induces a re-

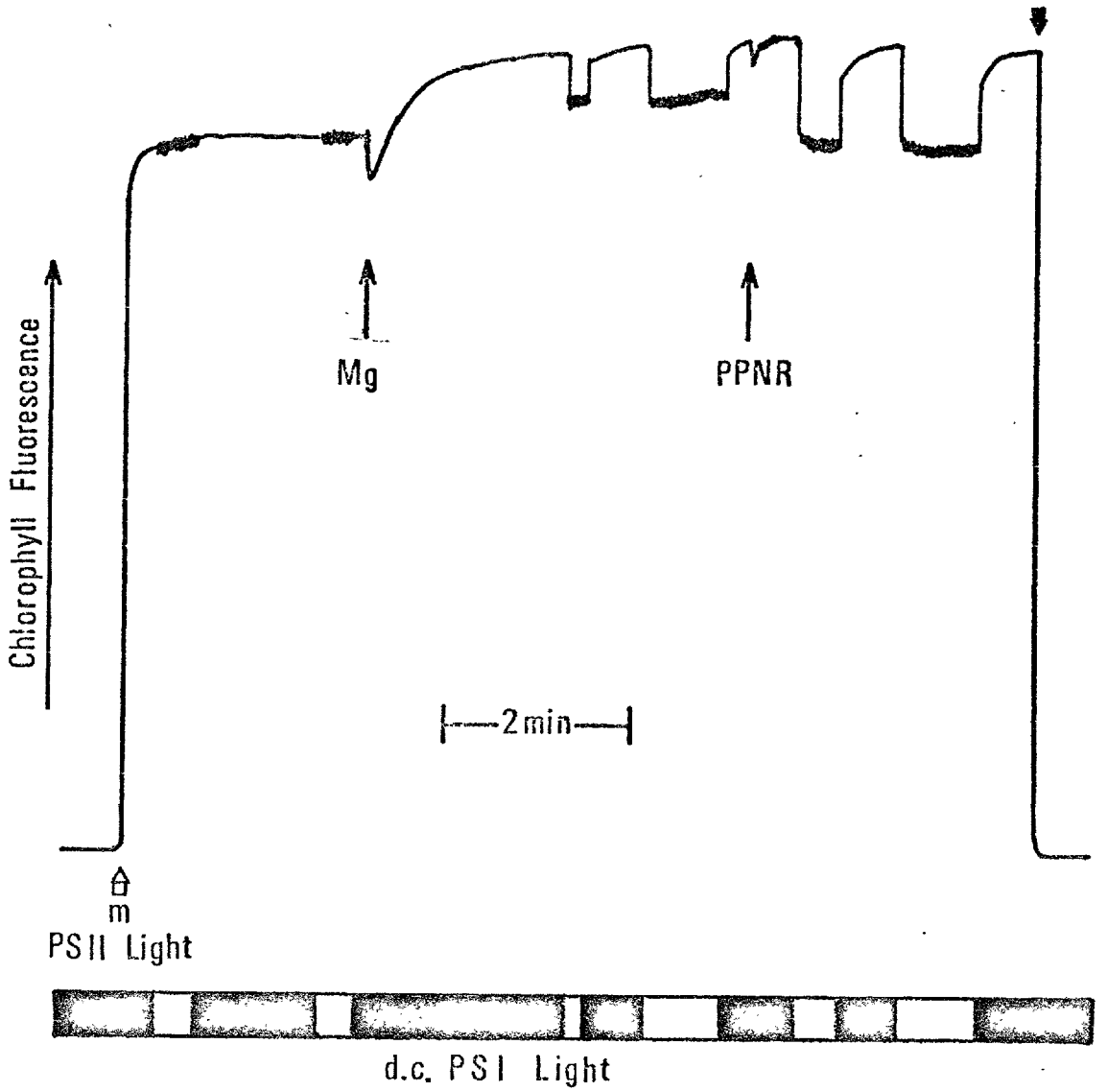


Fig. 3.17 Effect of 10 mM Mg^{2+} on the enhancement phenomenon observed in uncoupled broken chloroplasts. Broken chloroplasts (2o µg ml^{-1}) were initially suspended in assay medium containing 0.1 µM nigericin to create the low fluorescence state of the membranes. Note the inability of d.c. PSI light (lower open bar) to quench modulated fluorescence excited by a modulated PSII light until 10 mM $MgCl_2$ was added to the suspending medium. Other addition, PPNR (unpurified sample, approximately 50 µg protein ml^{-1} final concentration). See Section 2.2.3 for other experimental conditions.

oxidation of Q (seen as rapid quenching of fluorescence). These antagonistic effects of PSII and PSI lights on the Q-dependent yield of fluorescence represent another manifestation of the enhancement phenomenon. Under the conditions used in this experiment, 5 mM divalent or 100 mM monovalent cations are required in the medium in order to detect enhancement in broken chloroplasts. In the absence of these levels of cations, no enhancement was detectable, indicating that in this low salt situation, the blue light appears to excite PSII and PSI more equally. In this situation, Q remains largely oxidised. These results are consistent with the hypothesis that 5 mM Mg^{2+} or 100 mM monovalent cations inhibit spillover of excitation energy from PSII to PSI. This spillover normally equalises the relative distribution of blue light reaching each photosystem even though these wavelengths of light are apparently absorbed more by PSII than PSI.

In addition to cations, Sane and Park[210] have proposed that an unidentified 'enhancement factor' present in unpurified preparations of ferredoxin ('Photosynthetic Pyridine Nucleotide Reductase' or PPNR) also has to be added back to broken chloroplasts in order to observe enhancement clearly. On addition of PPNR to broken chloroplasts in the presence of Mg^{2+} (5 mM) it was indeed observed that enhancement, as viewed by a decrease of PSII fluorescence in PSI light was approximately doubled in broken chloroplasts (Fig. 3.17). However, similar effects were observed on adding MeVi at low concentrations to such chloroplasts. The increase in enhancement in this instance is therefore probably due to the addition of MeVi or PPNR in the role of electron acceptor to PSI rather than 'enhanced factor'. In the absence of added PPNR or MeVi the ability of PSI to reoxidise Q was probably limited by the lack of electron acceptor to PSI.

The antagonistic effects of PSII and PSI light on the redox state

of Q were then used to investigate whether changes in enhancement occur during high energy state quenching of broken chloroplasts pretreated with divalent monovalent cations in the absence of DCMU. As shown in Figure 3.18, such broken chloroplasts showed well marked enhancement effects, but little overall slow quenching of fluorescence. This was probably due to the rather low intensities of PSI and PSII light used (c.f. Fig. 3.1). Maximal high energy states could, however, be generated by subjecting the sample to a few minutes of high intensity illumination. During this period, however, measurements of enhancement were not possible since such illumination saturated electron transport. Following such a period of high intensity illumination, the yield of fluorescence induced by the weak, modulated PSII light was depressed, and enhancement decreased to a low level. The recovery of both fluorescence and enhancement then occurred with slow kinetics typical of reversal of high energy state fluorescence quenching in dark or weak illumination (Fig. 3.18). No such fluorescence changes were observed in broken chloroplasts preincubated in low levels of monovalent cations only. Uncouplers abolished the slow quenching and enhancement changes after high intensity illumination. Figure 3.19 also shows that identical phenomena were observed with intact chloroplasts. Again, the relatively low modulated illumination was too weak to significantly bring about slow fluorescence yield changes. Following a few minutes of intense illumination, however, the yield in weak light was decreased as was enhancement, both phenomena recovering with slow (approximately 5 minutes) kinetics.

3.2.7. Relationship of slow fluorescence quenching in isolated chloroplasts with state I/II changes in vivo

The cation sensitive quenching of variable fluorescence on generating a high energy state in broken chloroplasts was shown to be associated with

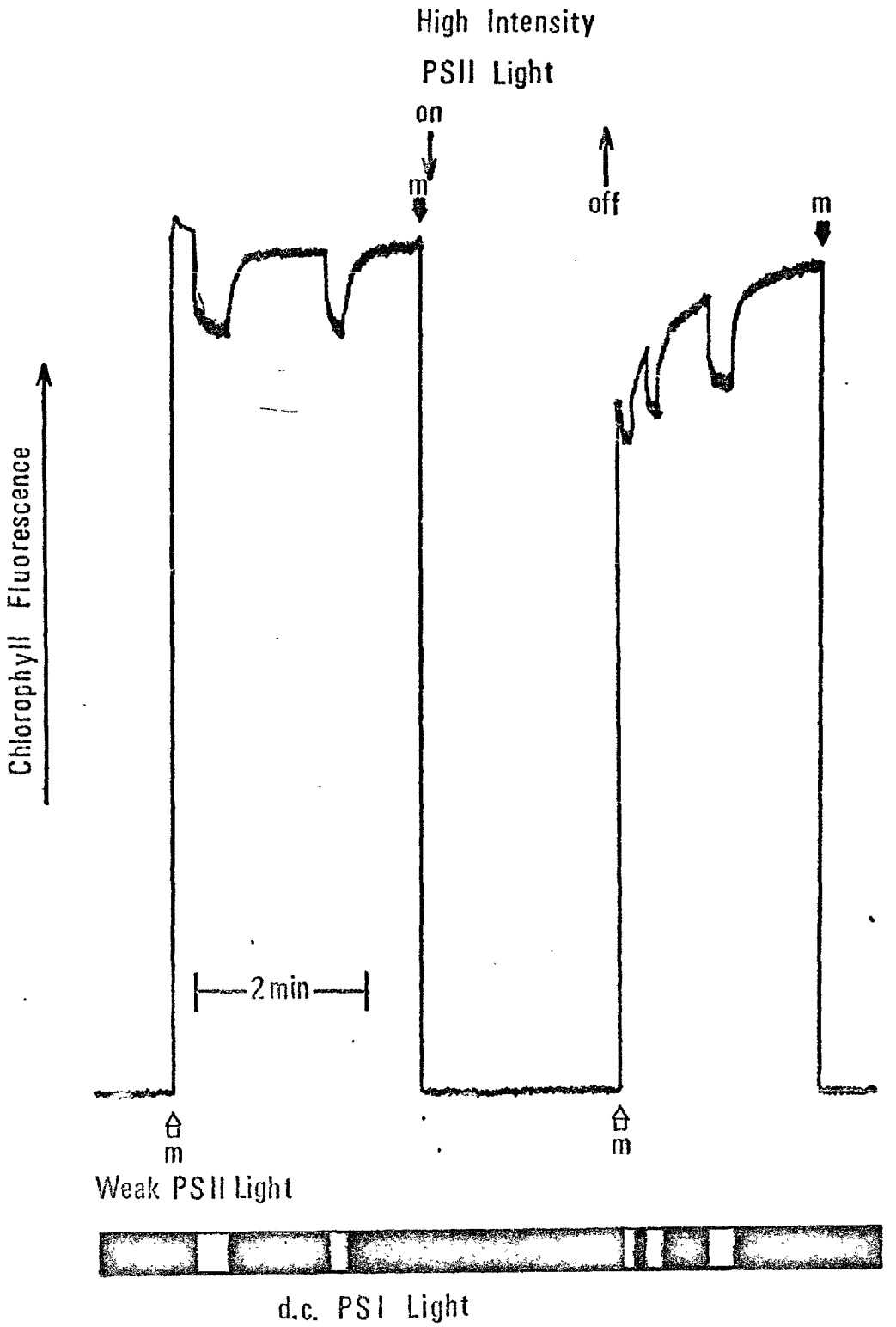


Fig. 3.18 Effect of high intensity PSII illumination on fluorescence and enhancement observed in coupled, broken chloroplasts suspended in assay medium containing 10 mM $MgCl_2$. Other conditions as for Fig. 3.17 except that nigericin was omitted from the suspending medium.

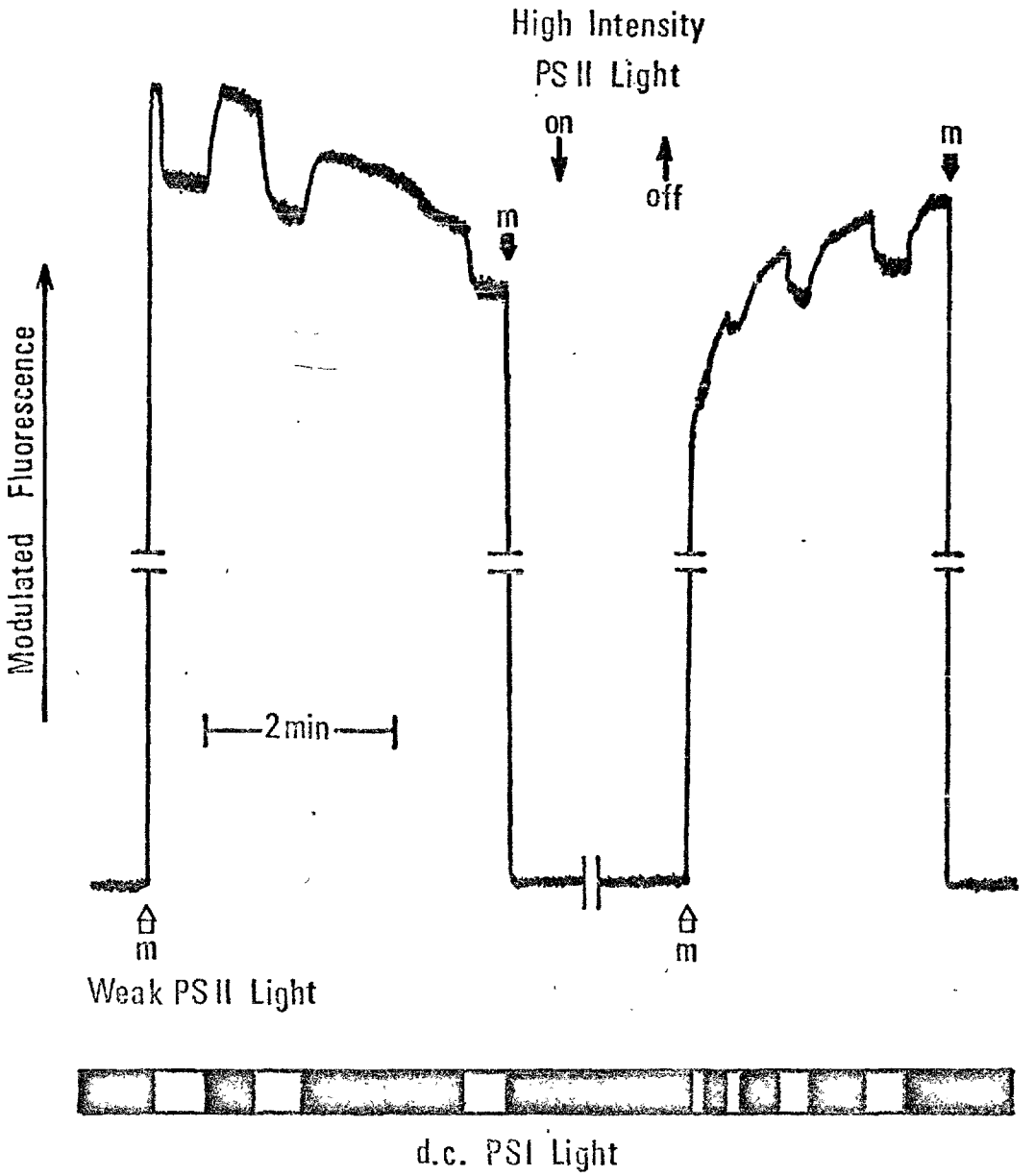


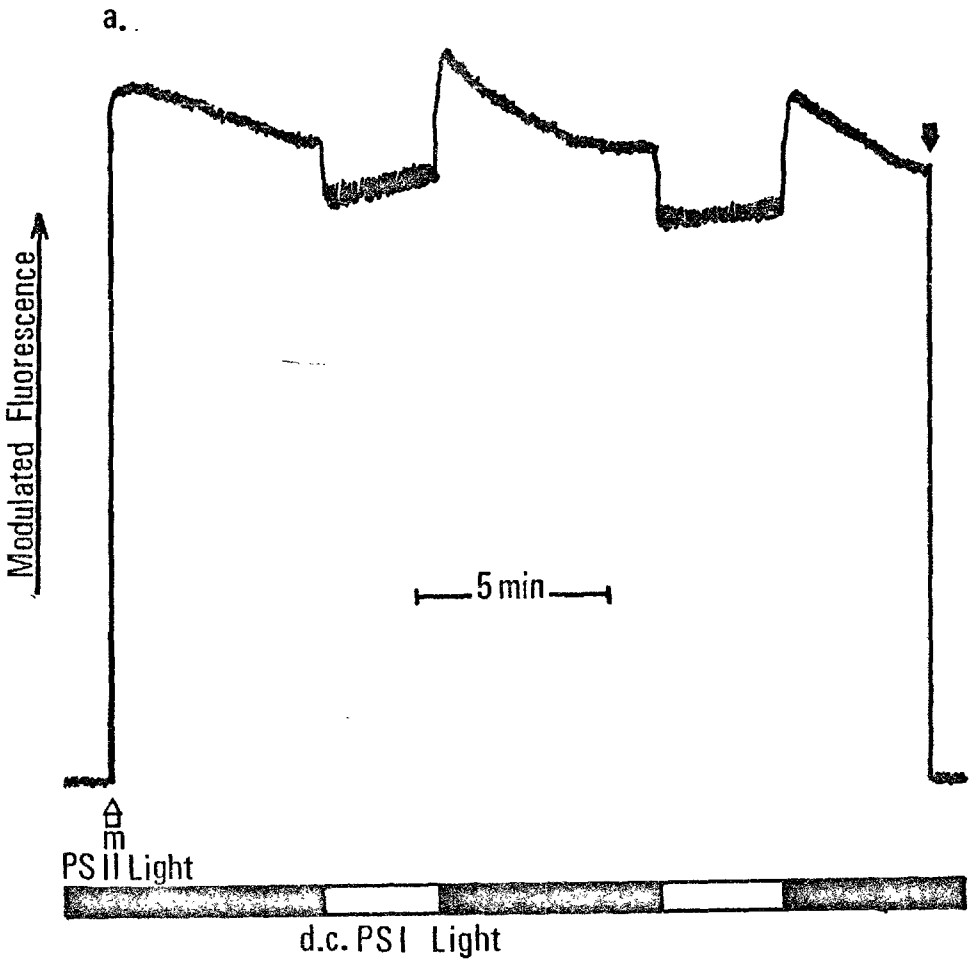
Fig. 3.19 Effect of high intensity PSII illumination on fluorescence and enhancement observed in intact chloroplasts. Intact chloroplasts (50% intact) were suspended in assay medium at 22 µg chlorophyll ml^{-1}. Other conditions as for Fig. 3.18 except that $MgCl_2$ was omitted from the suspending medium.

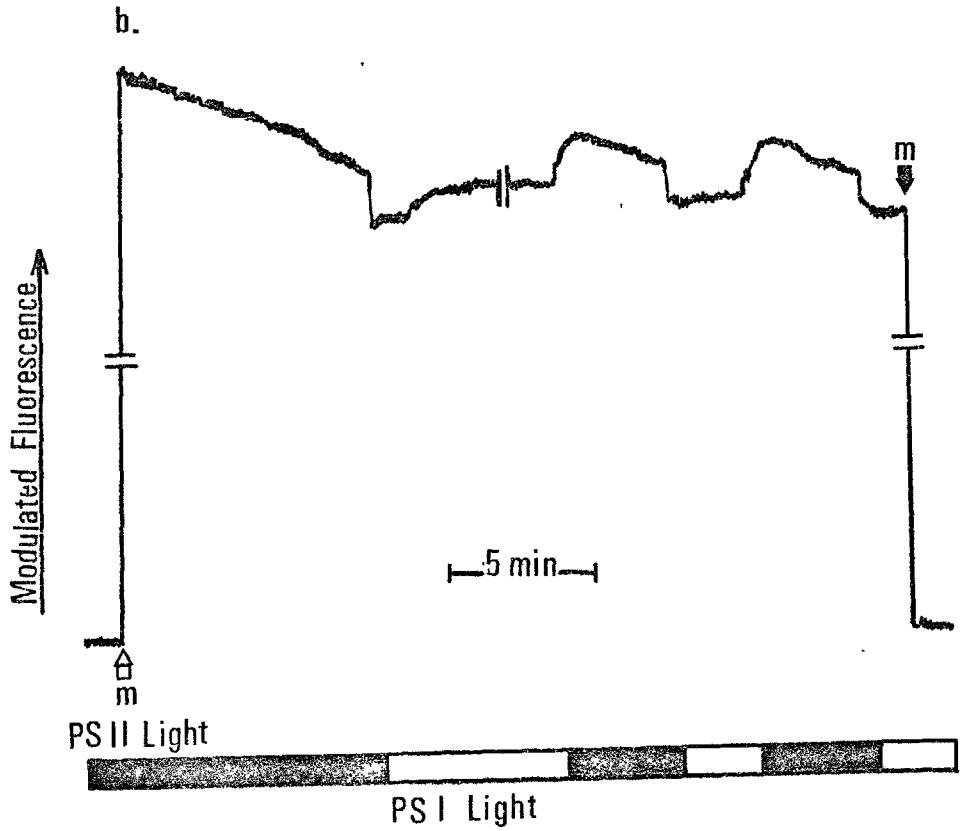
a decrease in enhancement and therefore clearly resembles the State I to State II transition observed in vivo.[155] Figure 3.20(a) illustrates that all of the features associated with State I to II transitions were observed in Chlorella using identical experimental conditions as for Figure 3.18. Apparently this weak intensity of modulated PSII light was sufficient to induce more extensive slow quenching of fluorescence in Chlorella than in isolated intact or broken chloroplasts from spinach. The slow quenching in vivo was associated with a decrease in enhancement as judged from the magnitude of Q quenching brought about by short periods of PSI light. Figure 3.20 also shows that the reverse process (i.e. State II to State I) can be observed in vivo on superimposing PSI light for longer periods. This is associated with a slow increase in fluorescence yield together with an increase in enhancement and is proposed to represent a decrease in spillover of excitation energy from PSII to PSI[156] as the algae adapts to illumination containing excess quanta absorbed by PSI. The state I/II transitions in vivo were fully reversible and could be repeated several times on the same sample of algae.

Although fluorescence and enhancement decreases associated with state I to state II changes were clearly and consistently demonstrable in isolated intact chloroplasts, the reverse process on superimposing PSI light for long periods was seen only infrequently in such preparations and not at all in broken chloroplasts pretreated with cations in the dark.

Figure 3.20(b) shows a preparation of intact chloroplasts which did show both state I to II and state II to state I changes in PSII and PSII plus PSI light respectively. The changes were small in comparison to the overall quenching of fluorescence which could be induced at high intensities of light, but were repeatable several times on the same sample.

Fig. 3.20 State I/II transitions as viewed by changes in chlorophyll fluorescence of (a) Chlorella, (b) intact chloroplasts (65% intact) suspended in assay medium at 22 µg chlorophyll ml^{-1} in the presence of 1 mM PGA. State I to State II occurred in PSII illumination and was characterised by a slow quenching of fluorescence and a decrease in enhancement, the latter observed as a rapid decrease in fluorescence on superimposing PSI light (d.c.) State II to State I occurred in PSII plus PSI light and was characterised by a slow increase in fluorescence and enhancement. For other experimental details, see Section 2.2.3.


a.
Modulated Fluorescence
5 min
m
PS II Light
d.c. PS I Light


b.
Modulated Fluorescence
m
5 min
m
PS II Light
PS I Light

Further studies of enhancement phenomena in intact chloroplasts in which both the oxygen evolution and fluorescence yield of PSII were monitored have been carried out in conjunction with Dr W.P. Williams and mainly performed by the latter.[211] These results also suggest that the stromal concentration of divalent cations may play an important a role in the mechanism of state I/II changes *in vivo* and are discussed more fully in the next section.

3.3. Discussion

The slow chlorophyll *a* fluorescence yield changes observed on illuminating isolated intact chloroplasts appear to be similar to those which occur during fluorescence induction (characterised by PSMT transients) *in vivo*.[121,122] The slow, light-induced decline in fluorescence observed with intact chloroplasts was not clearly differentiated into PS and MT phases, although a 'shoulder' was occasionally visible indicating that the kinetics may include two phases. This light-induced decline probably therefore, includes all the transients observed *in vivo*.

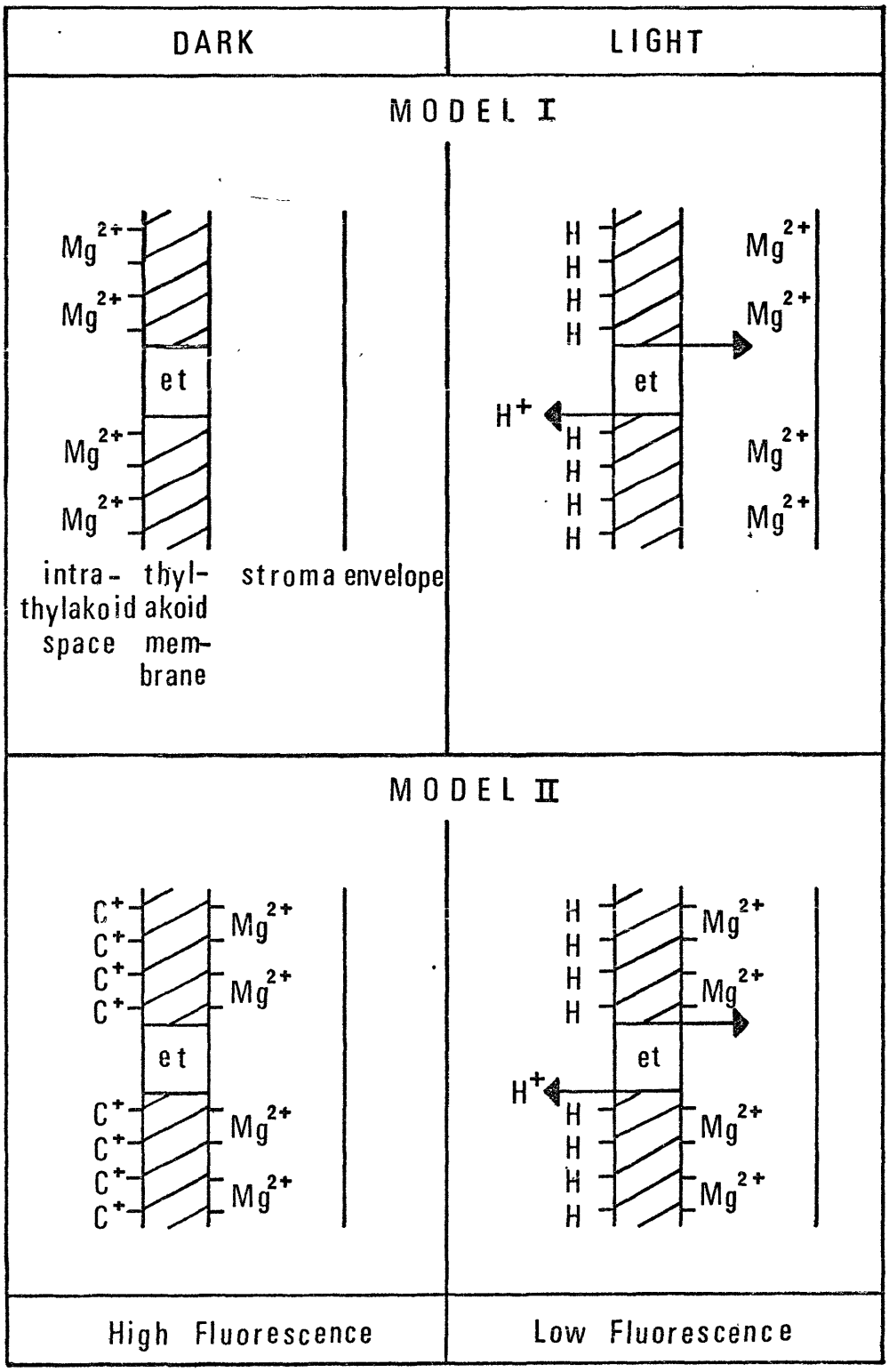
Uncoupler sensitivity of fluorescence quenching in such isolated organelles indicates that this process is mainly dependent on the generation and maintenance of the ΔpH component of the thylakoid high energy state. On removal of the outer envelope and suspension of the broken chloroplasts in media containing only low levels of monovalent cations, no such slow fluorescence changes are observed until cations at concentrations which induce the maximal increase in initial fluorescence yield are added to the chloroplast suspension in the dark.[194,196] It seems, therefore, that slow fluorescence quenching is dependent both on the high energy state and on a high stromal activity of cations within the isolated intact organelle. It seems that the outer envelope normally presents a barrier to the diffusion of these cations which are retained within the

organelle even after isolation and resuspension in media of relatively low ionic strength.[11,194,196]

Most of the results presented in the preceeding sections can be interpreted on one of two models depicted in Figure 3.21. Both models propose that the high energy state quenching of fluorescence essentially involves a reversal of the increase in yield induced by adding cations (5 mM divalent or 100 mM monovalent) to broken chloroplasts suspended in low levels of monovalent cations only. In the first of these (Model I, Figure 3.21) light-induced fluorescence quenching is proposed to be due to removal of cations from their sites of action. As originally proposed by Barber and Telfer[194] and Krause,[196] the high fluorescing state of dark adapted intact chloroplasts is due to the presence of metal cations associated with fixed negative charges on the inner side of the thylakoid membrane. This situation is a result of the high stromal activity of these cations within the organelle, and is not seen in broken chloroplasts until 5 mM divalent or 100 mM monovalent cations are restored to the preparation. On initiating coupled electron transport, protons pumped electrogenically into the intrathylakoid space displace metal cations associated with fixed negative charges on the inner side of the thylakoid membrane. These displaced metal cations then efflux across the thylakoid into the stromal space. Fluorescence quenching is caused by conformational changes in the thylakoid as cations are displaced from, or protons bound to the internal negative sites, and thus occurs concomitantly with the formation of the ΔpH gradient across the thylakoid. This process is reversed and fluorescence restored to the high yield on dissipating the transthylakoid pH gradient by uncouplers or termination of electron flow and associated proton pumping (darkness or addition of the electron transport inhibitor DCMU).

The second model (Model II, Figure 3.21) differs from the first in

Fig. 3.21 Two schematic models proposed to account for the slow quenching of chlorophyll a fluorescence observed on illuminating intact chloroplasts. Model I proposes that the high fluorescing state of dark adapted intact chloroplasts is created by the interaction of metal cations (probably Mg^{2+}) with fixed negative charges on the inner side of the thylakoid membrane. This occurs as a result of the high stromal activity of these cations (not shown). Initiation of light-induced electron transport and associated proton pumping results in displacement of Mg^{2+} to the stromal compartment and protonation of the internal thylakoid negative sites and this process brings about fluorescence lowering. Model II proposes that the high fluorescence of dark adapted intact chloroplasts is created by the interaction of stromal Mg^{2+} with fixed negative charges on the outer side of the thylakoid membrane. Light-induced fluorescence lowering is again proposed to result from protonation of fixed negative charges on the inner side of the thylakoid membrane, though in this case the nature of the inorganic cation displaced from the internal charges may be unimportant in terms of *in vivo* fluorescence lowering. Model II differs from Model I in that fixed negative charges on either side of the thylakoid membrane may influence *in vivo* chlorophyll fluorescence yield.


DARK
LIGHT
MODEL I
Mg2+
Mg2+
et
Mg2+
Mg2+
intra- thylakoid space
thyl- akoid mem- brane
stroma
envelope
H
H
H
H
H+
MODEL II
C+
C+
C+
C+
High Fluorescence
Low Fluorescence

that the sites of action of the high energy state and of cations which control fluorescence are not identical. In this model, high stromal levels of divalent or monovalent cations are proposed to interact at sites on the external (stroma facing) surface of the thylakoid to maintain this membrane in a configuration characterised by a high chlorophyll a fluorescence yield in the dark. Protonation of negative sites on the inner surface of the thylakoid membrane occurs on initiating light induced proton pumping, and this process results in a membrane conformational change which brings about the low fluorescing state. Disruption of the intact chloroplast into media containing only low levels of monovalent cations also results in a conformation change in the membrane which induces the low fluorescing state. This latter process is proposed to occur through cation control of fluorescence at the sites located on the external surface of the thylakoid. Low concentrations of monovalent cations do not interact with these sites in the same way as divalent cations or high concentrations of monovalent cations and the high fluorescence state of the membrane is consequently not seen. In this model, the proposed role of cations *in vivo* would be to maintain the membrane configuration that generates the high fluorescing state and allows high energy state effects to be expressed. The nature of the exchange cation in response to proton pumping is, in this case, unimportant.

Both of the above models require that intact chloroplasts maintain high stromal levels of cations *in vivo*. Experiments with cation specific ionophores (see Section 3.2) suggested that isolated intact organelles suspended in low salt buffer (containing no inorganic cations) maintained sufficient internal concentrations of Mg^{2+}, but not Ca^{2+} or monovalent cations to allow ionophore reversal of slow fluorescence quenching. This result implies that Mg^{2+} may be the main counter-ion to proton pumping within the organelle and that this process also controls the fluorescence

changes (Model I). According to Model II, the stromal level of Mg^{2+} in the dark is sufficient to satisfy the externally located thylakoid negative sites controlling fluorescence.

The finding that the apparent activity of monovalent cations in such organelles was low, whereas the measured internal concentrations before nigericin addition was high[205] is, however, both a striking and puzzling result and one that must be interpreted with care. It could be that the inability of nigericin (or valinomycin plus CCCP) to reverse fluorescence quenching of such chloroplasts in the absence of added K^+ is not due to low stromal activity of this cation, but to other reasons. It is possible for example that this antibiotic is unable to enter the organelle until small amounts of external K^+ are made available. This seems unlikely, however, bearing in mind the lipophilic nature of these antibiotics[84,85] and the fact that nigericin was used as the K^+ salt.

Alternatively, one must take account of the fact that nigericin (and valinomycin plus CCCP) might act at the outer envelope of the intact chloroplast to allow K^+ leakage. This certainly seems possible since this type of leakage was observed for divalent cations when ionophore A23187 was used in the presence of EDTA.

It seems that in broken chloroplasts reconstituted with 100 mM KCl, EDTA treatment removes residual Mg^{2+} (or Ca^{2+}) which is either in solution or is associated with the granal membranes. This residual divalent cation is sufficient to allow A23187 to exert its uncoupling properties and reverse fluorescence quenching in the absence of added Mg^{2+}. With intact organelles, the effect of EDTA on uncoupler sensitive fluorescence changes appears to be more complex. In the absence of EDTA, A23187 efficiently reversed slow fluorescence quenching presumably by inducing rapid $2H^+/Mg^{2+}$ exchange across the granal membrane and thereby collapsing the ΔpH gradient to return the chloroplast to the relaxed, high fluores-

cent state. In the presence of EDTA, however, not only was A23187 ineffective in this process, but the ability of any other treatment (darkness, nig + KCl, DCMU) to subsequently restore fluorescence quenching (i.e. in the presence of EDTA plus A23187) was also reduced. Under these conditions, it seems that A23187 may be acting mainly at the outer envelope of intact chloroplasts to encourage Mg^{2+} leak from the organelle. Since EDTA binds Mg^{2+} strongly, this would create a large concentration gradient for this divalent cation across the outer envelope which is made permeable in the presence of the antibiotic. Support for this comes from studies of OAA-promoted electron flow in intact chloroplasts suspended in low salt buffer.[205] A23187 initially uncoupled this electron flow, but addition of 1 mM EDTA to the external medium inhibited uncoupling, presumably because of Mg^{2+} depletion from the stroma.[205] That this process does not occur in the absence of EDTA may be explained by weak retention of Mg^{2+} by stromal proteins.

If A23187 plus EDTA does induce loss of stromal Mg^{2+} to the medium, then the reduced ability of other treatments to reverse fluorescence quenching of intact chloroplasts under these conditions further supports the contention that Mg^{2+} is the main cation involved in the control of fluorescence in vivo.

The small degree of dark and DCMU reversibility of quenched fluorescence which remains after A23187 plus EDTA treatment can partly be explained by the Q-dependent contribution to slow fluorescence yield changes. Even allowing for Q effects, a residual slow reversibility remained which was induced by nigericin plus KCl, but not nigericin alone, and which may be dependent on monovalent cations within the organelle. If this is the case, it is necessary to assume that stromal levels of monovalent cations may normally be quite high. The inability of nigericin alone to reverse slow fluorescence quenching of chloroplasts

in low salt buffer may therefore be due to the action of this ionophore at the outer envelope, causing leak of K^+ into the suspending medium. When low levels of K^+ are included in the suspending medium, nigericin acts mainly at the thylakoid membrane and utilises the internal K^+ levels of the intact chloroplast to effect uncoupling. Thus the ability of nigericin to induce leakage of K^+ from the organelle, if this is the case, would be dependent on the absence of K^+ in the external medium. Since nigericin catalyses H^+/K^+ exchange across biological membranes, this may not be entirely unexpected. The transport of the nigericin/K^+ complex out of the chloroplast must be balanced by an equal inward movement of protonated ionophore. Thus the ability of this antibiotic to dump K^+ in the outer medium in exchange for protons depends on the relative concentration of K^+ and H^+ in the external medium, and this process might well require a very low external K^+ concentration. This would especially be so at pH 7.6 where the H^+ concentration is also very low. An alternative way of looking at this problem is to consider the driving forces which determine nigericin induced leak from chloroplasts. Initially, a high stromal/low medium K^+ gradient would encourage K^+ transport out of the organelle in exchange for the uptake of protons. As this process continues, a ΔpH gradient in the opposite direction would be expected to be developed across the outer envelope. This would eventually be expected to halt K^+ efflux when the chemical potential of the pH gradient balances that of the K^+ gradient. Thus the extent of the nigericin facilitated K^+ efflux into the medium depends on the initial magnitude of the K^+ gradient across the envelope would be reduced as the external K^+ level is raised. This mechanism depends on a low proton permeability of the outer membrane. There is evidence that the intact chloroplast can maintain significant pH differentials between the stroma and the external medium.[2,11] Such a model may be tested by varying the external pH of the

medium. At low external pH, it might be expected that nigericin-induced leakage of K^+ may well be less dependent on the external K^+ concentration.

Overall, it can be concluded from investigations with cation specific ionophores that mainly divalent cations, probably Mg^{2+}, are involved in light dependent chlorophyll a fluorescence quenching observed in isolated intact chloroplasts. At this stage, however, it would be premature to dismiss a role of monovalent cations in this light induced fluorescence quenching phenomena. A conclusion on the involvement of monovalent cations in light induced fluorescence quenching will depend on future experiments involving careful analysis of the ionic content of isolated intact chloroplasts both before and after addition of ionophores to the suspending medium. Such experiments are shortly to be initiated in this laboratory.

Either of the models outlined in Figure 3.21 can account for the cation dependent reversible fluorescence changes observed in broken or isolated intact chloroplasts discussed above. The pH profile of the Mg^{2+} stimulated increase in chlorophyll fluorescence in DCMU poisoned broken chloroplasts suggests that negatively charged groups of pK around 5 are involved in this process. However, this result gives no information as to the location of these negatively charged groups on the thylakoid membrane. On decreasing the suspending medium pH below 6 units, these groups become protonated and at pH 4.5, only the low fluorescent state is seen in the presence or absence of 5 mM Mg^{2+} (or 100 mM monovalent cations). It is pertinent to note that the intrathylakoid space is generally assumed to contain buffers of pK around 5 to which most of the protons taken up in the light, on development of the high energy state, are thought to become bound.[65,78,79,97,100] It is possible therefore that such buffers may be equated to fixed negative charges on the inner side of the thylakoid membrane. The yield of fluorescence would depend

on whether Mg^{2+} or H^{+} are associated with these negative charges (Model I). However, the pH profile of cation stimulated fluorescence changes in broken chloroplasts may also reflect the protonation of groups controlling fluorescence on the outer side of the thylakoid membrane (Model II). If this is the case, it is doubtful whether this result has any physiological significance, since proton pumping results in alkalisation, and not acidification of the stromal compartment of the intact organelle.[2]

Recently, Jennings et al[202] have reported studies on light induced fluorescence quenching in isolated, broken chloroplasts which was not dependent on the transthylakoid high energy state. They found that such quenching was also independent of changes in the redox state of PSII. The fluorescence decline observed by Jennings et al[202] was stimulated by cations at higher concentrations than those required to induce the maximum initial yield of fluorescence of chloroplasts suspended in low levels of monovalent cations. Uncouplers stimulated this quenching at pH 9.2, whereas at pH 7.2 some uncouplers (CCCP, desaspidin) stimulated, but others (NH_4Cl, gramicidin) inhibited light induced quenching. They related their observations[202] to those observed in this laboratory[212,213] and concluded that slow fluorescence quenching was not driven by the developing transthylakoid pH gradient. They rather proposed that this process reflected an increase in radiationless de-excitation of chlorophyll excitation energy linked to membrane conformational changes associated with the coupling factor.

However, it seems unlikely that the uncoupler insensitive (irreversible) quenching of fluorescence observed by these authors[202] is related to reversible quenching reported in this thesis. Apart from the differences in uncoupler sensitivity, the first order kinetics observed by Jennings et al are clearly more rapid than the slower kinetics (see Figure 3.12), associated with high energy state effects on fluorescence in cation-

pretreated broken chloroplasts. Wraight and Crofts[159] had previously noted that Q independent reversible and irreversible quenching of chlorophyll fluorescence occurred in ferricyanide treated broken chloroplasts. They found that these two types of fluorescence quenching represented different processes, as judged by the differential uncoupler sensitivity and pH dependence. A more recent study by Sokolove and Marsho[214] has clearly shown that A23187 reversible fluorescence quenching of isolated, intact chloroplasts exhibits a much lower light intensity dependence than ionophore irreversible quenching. At light intensities of 80-100 Wm^{-2} (blue band illumination as used in the experiments reported in this thesis), fluorescence quenching was mainly reversible and displayed the slow kinetics typical of high energy state induced fluorescence changes. According to Sokolove and Marsho,[214] on increasing the light intensity ten-fold, quenching became irreversible and acquired the properties reported by Jennings et al.[202]

It appears therefore that irreversible fluorescence quenching reflects 'photoinhibition'[214] (damage to the chloroplasts at high light intensities) which may possibly involve photodestruction of the Mg^{2+} sensitive sites controlling chlorophyll a fluorescence. These deleterious effects of high light intensity on isolated chloroplasts probably have no importance in vivo, where mechanisms may exist to protect the in vivo chloroplast from photo-induced damage.[122]

It seems likely that the uncoupler sensitive reversible fluorescence quenching observed in isolated chloroplasts at lower light intensities reflects a more physiological response by the membranes to the light dependent development of a ΔpH gradient across the thylakoid. These reversible fluorescence yield changes may therefore be identified with similar changes extensively reported to occur in intact photosynthetic organisms.[121,122,132-136]

Slow, uncoupler sensitive fluorescence lowering also can occur in isolated intact or broken chloroplasts in the presence of DCMU when DAD is used to stimulate cyclic electron flow. Previous workers, using high concentrations of cofactor found that DAD[160] or PMS[161]-induced fluorescence quenching observed in broken chloroplasts was not affected by the presence of high concentrations of cations in the medium. These workers therefore dismissed any interrelationship between such high energy state quenching and cation effects on chloroplasts. However, the re-investigation of this process reported in Section 3.2.4. indicated that DAD-induced fluorescence quenching was strongly dependent on the levels of cofactor employed. At low concentrations of DAD (less than 12 μM) fluorescence lowering was very similar to that supported by endogenous non-cyclic electron flow in both intact and broken chloroplasts. In broken chloroplasts both types of fluorescence change showed (i) strong dependence on the presence of cations which increase the initial fluorescence yield, (ii) correlation with the extent of the ΔpH gradient, and (iii) a decrease in PSII fluorescence emission at 77°K relative to PSI. It seems therefore that cation dependent fluorescence quenching may occur on establishment of a pH gradient supported by either non-cyclic or cyclic electron flow.

Higher concentrations of DAD (greater than 12 μM) induced fluorescence quenching from either the higher or low fluorescence state of chloroplasts and which therefore might be due to other mechanisms as suggested by previous workers.[160,161] Clues to the mechanism by which this may occur are provided by the observation that the oxidised form of DAD at very high concentrations (1 mM) acts as a quencher of chlorophyll fluorescence in uncoupled chloroplasts at pH 4.5 but not pH 7.6.

Studies by Hauska and co-workers[215-217] have shown that cofactors such as DAD and PMS catalyse PSI dependent electron flow which is coupled to proton pumping by an artificial shuttle of protons across the thylakoid,

as outlined in Figure 3.2.2. The reduced form of DAD appears to be oxidised on the inner side of the thylakoid, and this results in the release of protons into the intrathylakoid space. The oxidised DAD moiety then passes through the thylakoid to the outer surface where re-reduction by PSI occurs and protons are taken up from the external medium. The cycle is completed on diffusion of the reduced DAD back through the membrane. On the basis of this model, the DAD induced fluorescence quenching can be explained as follows. Low concentrations of cofactor catalyse the establishment of a light induced ΔpH gradient across the thylakoid. This brings about a decrease in fluorescence quenching as negative sites on the inner side of the membrane become protonated (Model I or II). At higher concentrations of DAD, additional fluorescence quenching occurs as the oxidised form of cofactor accumulates at high local concentrations in the internal granal space. This results in direct quenching of chlorophyll excitation energy by DAD in a manner which is enhanced by the low intrathylakoid pH. Although both the direct (high concentrations) and indirect (low concentrations) DAD induced quenching phenomena are dependent on the ΔpH gradient, it is doubtful whether that seen at high cofactor concentrations has any 'physiological' significance. For example, Papageorgiou and co-workers[122,162,163] have recently presented evidence that a similar 'non-physiological' mechanism may account for PMS stimulated fluorescence quenching which also implicates a role of molecular oxygen in this process. They found that PMS dependent quenching in broken chloroplasts was reduced by half on making the sample anaerobic. The full extent of quenching could be restored by simply reequilibrating the anaerobic sample with air.[122,162] It was therefore concluded by these authors that protonation of the thylakoid membrane which occurred during PMS-induced proton uptake enhanced the diffusion, not only of PMS, but also of O_2 to the chlorophyll bed where these agents then quenched chlorophyll

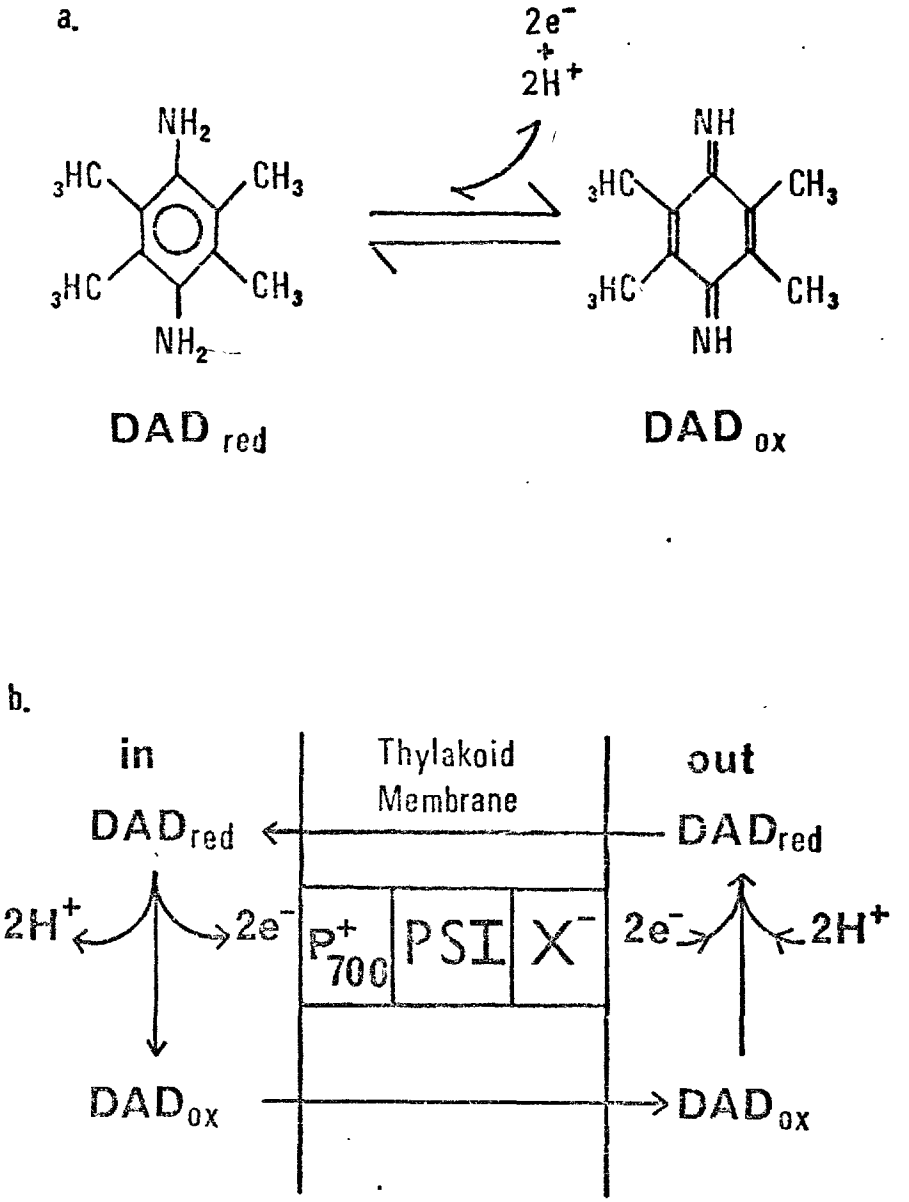


Fig 3.22 (a) Oxidation and reduction of diaminodurene (DAD) emphasising that release and uptake of protons are involved in the process. (b) Schematic representation of the mechanism by which DAD is thought to catalyse cyclic electron flow and associated proton pumping in illuminated chloroplasts treated with DCMU. Reduction of the oxidised form of DAD is proposed to occur on the outer side of the thylakoid membrane, and reoxidation of the reduced form on the inner side of the membrane. This would result in the net translocation of two protons across the thylakoid membrane per pair of electrons per cycle.

excited states. Papageorgiou has implicated such a mechanism in the PS fluorescence induction transient *in vivo*. This phase is also severely inhibited under anaerobic conditions, and is associated with an increased oxygen evolution by algae.[122] It is visualised that high light intensity induces maximal ΔpH gradients *in vivo*, and this enhances parasytic quenching of chlorophyll excitation energy by molecular oxygen according to the following scheme.[122]

$$Chl^{*} + O_2 \rightarrow Chl + O_2^{*} \qquad \text{Eq. 3.1}$$

$$O_2^{*} + \text{carotenoid} \rightarrow \text{epoxy carotenoid} \qquad \text{Eq. 3.2}$$

$$\text{Epoxy carotenoid} + 2H^{+} + 2e^{-} \rightarrow H_2O + \text{carotenoid} \qquad \text{Eq. 3.3}$$

Such a mechanism may constitute a 'safety valve' *in vivo* where potentially destructive excitation energy resulting from light absorbed, but not utilised (at and above saturating intensities) may be safely dissipated.

It is not clear whether such a mechanism operates in isolated intact or broken chloroplasts. Direct quenching of fluorescence by molecular oxygen (or other diffusible quenchers) essentially involves an increase in radiationless de-excitation of singlet chlorophyll. Barber and Telfer[194] and Telfer *et al*[218] found that part of the slow fluorescence quenching at room temperature observed in illuminated intact chloroplasts was associated with a decrease in emission at 685 nm and 730 nm at low temperature($77^{o}K$). Since these two emission peaks are thought to represent fluorescence from PSII and PSI respectively, this would indicate that an overall decrease in the quantum yield of fluorescence of both photosystems had occurred. This is consistent with an overall increase in radiationless de-excitation brought about by parasytic quenching of singlet chlorophyll by oxygen. The observation that slow fluorescence quenching in isolated intact chloroplasts (and broken chloroplasts pretreated with appropriate levels

of cations) requires relatively high light intensities is also consistent with this hypothesis.

However, the fact that no slow fluorescence quenching is seen in broken chloroplasts suspended in low levels of monovalent cations indicates that no high energy state enhanced O_2 quenching occurs under these conditions. It is possible of course that low divalent or monovalent levels of cations enhanced the permeability of the thylakoid to such quenchers as PMS and O_2 and thereby allow these agents to act more efficiently in the presence of the high energy state. However, these levels of cations would be expected to decrease the initial fluorescence yield of broken chloroplasts (increased access of O_2) whereas experimentally the initial fluorescence is increased. It seems unlikely therefore that O_2 quenching of fluorescence occurs to any appreciable extent in isolated chloroplasts.

The increase in initial yield of fluorescence induced by 5 mM Mg^{2+} or 100 mM K^+ in broken chloroplasts, and the subsequent slow fluorescence yield quenching seem to be better explained by changes in the distribution of light energy between the two photosystems. It was shown in Section 3.2.6. that for uncoupled chloroplasts in the presence of low levels of monovalent cations (low fluorescing state), PSI had little ability to re-oxidise Q via the electron transport chain when PSI light was superimposed on weak, blue band modulated light. This seems to indicate that, under these conditions, the modulated light excites both PSII and PSI about equally and Q therefore is largely oxidised. However, on adding 5 mM divalent cations (or 100 mM monovalent cations) to the medium, the resulting increase in overall fluorescence was accompanied by a relative increase in the excitation of PSII compared to PSI as judged by the ability of PSI light to oxidise the excess reduced Q. It appears therefore that in the presence of these cations, modulated blue light excited PSII

more than PSI. This is consistent with the hypothesis that 5 mM divalent or 100 mM monovalent cations inhibit spillover of absorbed light energy from PSII to PSI, resulting in increased yield of fluorescence (which at room temperature originates mainly from PSII).[157] It appears that in blue illumination, 5 mM divalent or 100 mM monovalent cations are required to observe enhancement in broken chloroplasts. This was confirmed by direct measurements of modulated O_2 evolution in the presence of $NADP^+$, performed on a modulated oxygen electrode in conjunction with Dr W.P. Williams of Chelsea College, London. It was found that the rate of O_2 evolution observed in modulated blue light was not enhanced on superimposing PSI light until the medium contained 5 mM divalent or 100 mM monovalent cations. In the presence of 30 mM monovalent cations which satisfy the additional cation requirement of ferredoxin functioning[179,181] no enhancement was observed.

Overall, the results indicate that divalent or high concentrations of monovalent cations increase the yield of PSII fluorescence by inhibiting spillover of excitation energy from PSII to PSI as concluded by others.[128,157,172,174,177,194] It was also shown in Section 3.2.6 that broken chloroplasts in the presence of these cations (like isolated intact chloroplasts) initially show enhancement in weak modulated blue light. However, after high energy state quenching of fluorescence had been induced by strong actinic illumination, the yield of fluorescence on removing this high intensity light was low and enhancement was small. This result indicates that the quenched fluorescence level on generating a ΔpH gradient across the thylakoid is similar to the effects seen when chloroplasts are initially suspended in only 10 mM monovalent cations to create the low fluorescing state. It appears that the high energy state reverses the effects of 5 mM divalent or 100 mM monovalent cations on broken chloroplasts, and again increases spillover from PSII to PSI.

This may occur by a mechanism proposed either in Model I or Model II. Such changes cannot be explained by a DAD or PMS[122] type mechanism proposed to account for light induced fluorescence quenching observed at high concentrations of these cofactors. It was clearly shown that in the presence of high levels of DAD (250 μM) and the light-induced high energy state of chloroplasts, the quantum yield of PSI (as judged by low temperature spectra) was reduced more than that of PSII. This would result in an overall decrease in chlorophyll fluorescence at room temperature together with an increase in enhancement. Experimentally, however, such a correlation was not seen. The results seem to indicate that in vivo, the MT phase of fluorescence induction,[122] which is associated with a decrease of both fluorescence and enhancement may be controlled by the magnitude of the in vivo ΔpH gradient across the thylakoids. These changes thus constitute the State I to State II transition.[155] If this is so, it is necessary to think of a mechanism by which the State II to State I transition occurs. This process which occurs slowly (5 minutes) in the presence of PSI illumination results in an increase in fluorescence and return to the high enhancement condition. This implies that a decrease in the magnitude of the ΔpH gradient occurs in vivo in PSI light. However, it might be expected that the activation of cyclic electron flow in such illumination would result in increased proton pumping and therefore increase the extent of the transthylakoid pH gradient. In fact the State II to State I associated increase in enhancement in PSI light was clearly observed in vivo using Chlorella, but only poorly so using isolated intact chloroplasts and not at all with broken chloroplasts pretreated with 5 mM Mg^{2+} or 100mM K^{+}. This might indicate that the mechanism of this change is more subtle than the opposite State I to State II transition and perhaps is more susceptible to inactivation on isolating chloroplasts from the leaf. Even in vivo

it has recently been shown[218] that observations of enhancement and State I/II transitions show great variability in the extent and shape of the signals depending on the age of the algal culture, state of diurnal rhythm, etc.

Measurements of enhancement by PSI light of O_2 evolution supported by PSII enriched illumination have recently been made with intact chloroplasts using the modulated oxygen electrode techniques described above and available at Chelsea College. Experiments, mainly performed by Dr W.P. Williams and colleagues[211] did not correspond to the 'classic' State I/II transitions observed by Bonnaventura and Myers. However, characteristic changes in the enhancement of O_2 evolution were evident which seemed to depend mainly on the equilibration and subsequent degradation of the intact organelles on the bare platinum electrode surface. During the latter phase of this process, PSI illumination induced slow changes of O_2 yield which were reminiscent of the State II to State I transition and appeared to correlate with a light dependent increase in the stromal levels of Mg^{2+}.[211]

In order to discuss the mechanism of the State I/II changes in molecular terms, it is necessary to choose between the two models presented in Figure 3.21. Although most of the data so far presented are consistent with either model, there are indications that Model II is better able to accommodate the detailed findings of some experiments. For example, it was found that increasing the concentration of Mg^{2+} in the suspending medium from 3-30 mM did not inhibit DAD stimulated light induced fluorescence quenching in broken chloroplasts when extremely limiting concentrations (4 μM) of cofactor were employed (see Section 3.2.4). This may suggest that protons taken up into the intrathylakoid space do not directly compete with metal cations for the sites controlling fluorescence. Such a competition between H^+ and Mg^{2+} is

predicted by Model I and should be observed at low DAD concentrations where the extent of light induced fluorescence quenching was observed to be small. The reduced extent of fluorescence quenching under these conditions would be due to the establishment of an equilibrium situation in steady state illumination in which a fraction of the internal fluorescence controlling sites are protonated and the remaining sites are associated with Mg^{2+}. Model I predicts, therefore, that an increase in the Mg^{2+} content of the suspending medium should alter this light induced equilibrium in favour of Mg^{2+} binding to the negative sites and thereby inhibit the extent of fluorescence quenching.

The fact that increased levels of Mg^{2+} in the suspending medium of broken chloroplasts pretreated with 4 μM DAD did not inhibit the extent of fluorescence quenching is more consistent with Model II than Model I. According to Model II, H^{+} taken up by chloroplasts and Mg^{2+} present in the external medium are proposed to act at different sites to effect fluorescence changes, and thus no direct competition between these ions is predicted.

The experiments reported in the next section were designed in order that a more conclusive choice could be made between Models I and II. The results to be presented also yield useful information as to the physical mechanism by which cations interact with the thylakoid membrane.

SECTION 4

THE MECHANISM OF CATION-INDUCED CHLOROPHYLL a FLUORESCENCE CHANGES IN ISOLATED, BROKEN CHLOROPLASTS

4.1. Introduction

4.1.1. Thylakoid structural changes and chlorophyll a fluorescence

In Section 3 it was shown that the addition of 5 mM divalent or 100 mM monovalent cations to broken chloroplasts suspended in media containing only low levels of monovalent cations resulted in an increase in room temperature chlorophyll a fluorescence. This increase in fluorescence (which originates mainly from PSII[121,122] seemed to be due at least in part, to a decrease in spillover of absorbed excitation energy from PSII to PSI, as suggested by others.[128,157,161,169,172,174,177,194] Two models were presented to account for these observations. Both models proposed that the presence of 5 mM divalent or 100 mM monovalent cations in the medium resulted in an association of these cations with fixed negative charges either on the inner (Model I) or outer side of the thylakoid membrane (Model II). The interaction between cations and the fixed negative sites on the thylakoid may somehow induce membrane conformational changes which control fluorescence and spillover.

However, any mechanistic explanation for cation induced changes in in chlorophyll fluorescence must also account for observations originally made by Cross and Hess[172] (and confirmed by others[173]). These authors found that the condition of minimum fluorescence and maximum spillover of DCMU treated chloroplasts required low concentrations of monovalent cations in the medium. Broken chloroplasts washed and resuspended in sucrose containing sufficient tris base to bring the medium to pH 8.0 were characterised by a high initial yield of fluorescence. This high yield was then decreased

to the low level on adding a variety of monovalent (but not divalent cations) up to a concentration of 10 mM.[172] The low fluorescence level could then be restored to the high yield on further adding 5 mM divalent or 100 mM monovalent cations as found previously by other workers.[157,168-171,174,194,196]

Gross and Hess concluded from measurement of low temperature emission spectra that the antagonism displayed by low concentrations of monovalent and divalent cations on the fluorescence yield of broken chloroplasts suspended in a cation-free medium were due to changes in spillover of excitation energy between PSII and PSI.[172]

Such spillover may well occur via a Förster-type mechanism of inductive resonance energy transfer between excited PSII chlorophylls (donor molecules) and ground state PSI chlorophylls (acceptor molecules).[219] Such a mechanism depends both on the distance and mutual orientation as well as the degree of overlap between fluorescence emission and absorption spectra of donor and acceptor molecules, respectively.[29,219] It seems likely that changes either in the distance or mutual orientation of chlorophylls of PSII and PSI could account for the cation induced changes in fluorescence and spillover. The most obvious way in which cations might alter the spatial arrangement of chlorophyll embedded in the thylakoid is through cation induced structural changes in the membrane itself. Two kinds of cation induced membrane structural (conformational) change can be conceived. Cations may effect large scale structural changes which physically alter the distance between PSII or PSI complexes.[220] Alternatively, small scale microconformational changes might occur which alter the distance or orientation of only a few, key chlorophyll molecules which link PSII and PSI.[221] Seely[221] has produced a computer model showing that such a change in orientation may involve as little as six chlorophyll molecules properly positioned between a highly ordered PSII and PSI complex containing 600 chlorophylls.

Divalent and monovalent cations have been found to affect various aspects of the structure of isolated broken chloroplasts. These will be reviewed in the following few sections in regard to the possible involvement of such structural changes in the cation induced effects on chlorophyll fluorescence.

4.1.2. Thylakoid stacking

As shown in Figure 1.1, much of the thylakoid lamellae system within the isolated intact chloroplast (and *in vivo*[7,8]) occurs as appressed discs, termed grana lamellae, which are found stacked together in the form of a pile of coins. Izawa and Good[222] first noted that the stacking of grana was dependent on the cation constitution of the suspending medium when broken chloroplasts were isolated from the leaf. This was confirmed by Murakami and Packer.[223] They showed that grana stacking was preserved by isolating chloroplasts in media containing 150 mM Na^+ but was not observed on resuspending the broken chloroplasts in distilled water. In this latter case, the membranes became discrete and swollen. Addition of 150 mM Na^+ to such unstacked lamellae caused the thylakoids to restack. Similar results were seen when divalent cations at lower concentrations (80 mM) were used in place of 150 mM monovalent cations. They also found that the unstacked membrane configuration corresponded to a low fluorescence yield of chloroplasts, whereas the high yield was found in stacked thylakoids.[223] These fluorescence changes appeared to be due to spillover changes as judged by studies of relative PSII and PSI emission at low temperatures.

These authors[223] argued that any electrostatic attraction between charged groups on the thylakoid surface would be weakened by the presence of electrolytes in the medium. They concluded therefore that the forces between thylakoids responsible for stacking were largely hydrophobic in

nature. Hydrophobic interactions between membranes would be strengthened in the presence of high concentrations of salts in the medium, and this caused membrane stacking. They further proposed[223] that the increased hydrophobic environment between stacked membranes brought about the increased fluorescence yield of such chloroplasts.

A later study by Gross and Prasher[224] indicated that the dependence of grana stacking on the ionic composition of the medium was more complex than that found by Murakami and Packer.[223] Gross and Prasher[224] found that the unstacked configuration of DCMU treated thylakoids depended on the presence of monovalent cations at low concentration in the suspending medium. In the absence of any added cations, but in the presence of an uncharged osmoticum (sucrose), the membranes of isolated, broken chloroplasts were initially stacked. Addition of 3 mM monovalent cations caused the membranes to unstack, and this could be prevented by the presence of 1 mM divalent cations in the medium. The apparent conflict between the results of Gross and Prasher[224] and Murakami and Packer may be due to the omission of an osmoticum from the media used by the latter authors, thereby adding osmotic stress to the membranes initially suspended in the absence of other cations.

The work of Gross and Prasher[224] indicates that both chloroplast fluorescence and membrane stacking show a remarkably similar dependence on the levels of cations in the suspending media. Low levels of monovalent cations are required to observe both the low fluorescence (maximum spillover) and unstacked configurations. High fluorescence and membrane stacking are observed in the absence of added cations or in the presence of low levels of divalent or high levels of monovalent cations. These authors dismissed the idea that electrostatic interactions between grana membranes controlled the fluorescence and stacking changes. They argued that any increased electrical repulsion between membranes caused by the unmasking

of fixed negative charges on reducing the ionic strength of the medium could not account for the stacked configurations observed in media of essentially zero ionic strength.[224] However, no alternative explanation was offered and the results stand largely unexplained.

4.1.3. Thylakoid volume and membrane thickness

It has long been known that the thylakoid membrane can act as an osmotic barrier to certain uncharged molecules such as sucrose. Gross and Packer[225] had found that the intrathylakoid volume of broken chloroplasts appeared to be inversely related to the osmotic strength of the suspending medium in different concentrations of sucrose. These volume changes appeared to follow the Vant-Hoff law, indicating that they were purely osmotic in nature. Such volume changes were explained as the gain or loss of intrathylakoid water from broken chloroplasts in response to differences in osmotic strength between the intrathylakoid space and outer suspending medium. Purely osmotic volume changes bear no relation to cation induced fluorescence yield changes.

However, cations appear to induce changes in both the volume of thylakoids and the thickness of the thylakoid membrane by non-osmotic mechanisms.[173,220,224,225] These cation induced changes in chloroplast structure have often been studied by optical techniques such as the intensity of light scattering at 90° (90° light scattering) or 180° (apparent transmission or absorption changes) to the measuring beam.

The use of these optical techniques as probes of thylakoid structure has been justified by Murakami and Packer by studies of broken chloroplasts maintained in high concentrations of monovalent cations.[13] Under these conditions the thylakoids remained stacked throughout the experiments. They found that illumination of coupled, broken chloroplasts resulted in proton uptake into the intrathylakoid space.[13] The extent of proton uptake

appeared to correlated well with an increase in 90° scattering of 546 nm light. Microdensitometric analysis of electron micrographs indicated that 90° light scattering changes also correlated with a decrease in the thickness of the walls of thylakoids within a grana stack.[13] They proposed that protonation of the inner side of the thylakoid reduced the net surface charge, and that this resulted in an increased attraction between hydrophobic regions within the membrane. This process caused the decreased thickness of the membrane.[13] The 90° light scattering appeared to be a reliable monitor of this process.

The decrease in membrane thickness (and increase in 90° light scattering) also occurred under conditions where the gross thylakoid volume either shrunk or swelled on illumination.[13] Light-induced shrinkage occurs in media containing weak acid anions such as acetate and appears to be due to loss of these anions (as protonated acid) from the thylakoid space.[12,226] Light dependent swelling of thylakoids occurs in high concentrations of highly dissociated anions such as Cl^{-} and is thought to result from influx of anions in response to electrogenic proton uptake.[12,98] The apparent absorption of 546 nm light was observed to increase when membranes shrunk in the light and to decrease when light induced swelling of broken chloroplasts was induced. The apparent absorption changes were found to be more closely related to the spacing between the internal walls of the thylakoid and, under these conditions were concluded to act as a probe for thylakoid volume.[13]

It should be noted that proton uptake on illuminating broken chloroplasts is associated with quenching of chloroplast fluorescence. This high energy state quenching of fluorescence therefore may correlate with a decrease in thylakoid membrane thickness, but not with light induced volume changes.

Addition of cations to broken chloroplasts also induces changes in

membrane structure related to thylakoid volume and membrane thickness.[173,220,224,225] Gross and Packer[225] found that addition of monovalent cations at high concentrations (up to 400 mM) induced thylakoid shrinkage of broken chloroplasts as detected by decreases in the absorbance ratio $A_{\frac{680}{546}}$, pellet weight and pellet volume. On the basis of ionic strength, divalent cations were found to be much more effective than monovalent cations at inducing this light-independent chloroplast shrinkage. It seems, therefore, that under these conditions, cations effect shrinkage of broken chloroplasts by non-osmotic mechanisms. However, it is unlikely that such non-osmotic shrinkage is related to cation induced chlorophyll fluorescence changes. Gross and Prasher[224] found that similar shrinkage was induced by addition of divalent cations to chloroplasts suspended in sucrose (plus sufficient tris to bring the medium to pH 8.0). Under these conditions the membranes are initially stacked and in the high fluorescing state, and addition of divalent cations has little effect on these latter phenomena.

Cations also appear to effect changes in the thickness of broken chloroplasts treated with DCMU to eliminate high energy state effects. When such broken chloroplasts are initially suspended in media containing low levels of monovalent cations, addition of divalent cations results in an increase in both chlorophyll fluorescence and 90^{o} scattering.[220] Furthermore, when care is taken to suspend broken chloroplasts in a cation free medium (high fluorescence), monovalent cations (less than 10 mM) bring about a decrease in both 90^{o} light scattering and fluorescence yield.[173] If 90^{o} light scattering is measuring the membrane thickness under these conditions, then it appears that cations can induce these structural changes in parallel with cation-induced fluorescence yield changes. A decrease in membrane thickness would correspond to an increase in chlorophyll fluorescence brought about by 5 mM divalent or 100 mM

monovalent cations. However, such a cation-induced decrease in thickness of the thylakoid cannot per se be the cause of the membrane conformational changes which increase fluorescence under these conditions. Murakami and Packer showed that light-induced proton uptake also resulted in a decrease in the thickness of the thylakoid membrane.[13] As shown in Section 3 of this thesis, light-induced high energy state formation in intact or broken chloroplasts results in quenching and never an increase in fluorescence yield.

Murakami and Packer have also found that protonation of broken chloroplasts brought about by lowering the external pH of the medium, both improves stacking and decreases the thickness of the thylakoid membrane.[13] Again, such protonation of broken chloroplasts is associated with a lowering of chlorophyll fluorescence (see Section 3.2.4) and not an increase in yield.

It must be concluded, therefore, that although cations can induce gross structural changes in broken chloroplasts which correlate with changes in fluorescence yield under many conditions, these structural changes per se are not the cause of the fluorescence yield changes.

4.1.4. Specific cation binding to thylakoid membranes

It has been concluded in the past that cations induce micro-conformational changes in the thylakoid membrane.[162,173,220,224] Such micro-conformational changes control the fluorescence changes but are not directly related to macroscopic structural features such as thylakoid stacking, volume and membrane thickness discussed in the previous sections.

Direct experimental evidence for the existence of cation induced micro-conformational changes in thylakoids which correspond to cation sensitive fluorescence changes is, however, somewhat meagre. Several authors[13,161,171] have shown that fixation of isolated broken chloroplasts with gluteraldehyde

prevents subsequent membrane conformation changes and also abolishes chlorophyll fluorescence changes induced either by cations or the light-induced development of the high energy state. These results indicate that fluorescence is somehow controlled by the particular structural state of the membrane.

An attempt has recently been made by Gross and Hess[227] to correlate both the fluorescence yield of broken chloroplasts and the structural state of the membranes (apparent absorption at 540 nm) with specific cation binding to the thylakoids. These experiments were carried out in the dark or with weak illumination and did not seem to involve high energy state effects on chloroplast fluorescence or structure. They found that apparent binding of Mg^{2+}, Ca^{2+} or Mn^{2+} to thylakoids occurred when these cations were added to broken chloroplasts washed and suspended in a cation-free medium. Although evidence was obtained for two different types of cation binding site, only one of these (apparent K_m 50 μM for Ca^{2+}, Mg^{2+} and Mn^{2+}) appeared to correlate with divalent cation induced structural changes in the membrane. Divalent cations under these conditions do not induce an increase in fluorescence which is already at the high yield for thylakoids suspended initially in a cation-free medium.[172] Analysis of Dixon plots (double reciprocal plots) also indicated that monovalent cations became bound to thylakoids suspended in cation-free media.[227] This apparent dissociation constant was, however, much higher (2-4 mM) than that for divalent cations under equivalent conditions. The presence of bound monovalent cations competitively inhibited subsequent divalent cation binding indicating that the same sites were probably involved for each type of cation. It should be noted that binding of monovalent cations did not induce structural changes[227] although under these conditions[172] they did decrease fluorescence. It seems unlikely, therefore, that the structural changes observed by these authors are related to fluorescence yield changes.

Atomic absorption analysis of the Ca^{2+}, Mg^{2+}, Na^{2+} K^{+} and Mn^{2+} content of such chloroplasts before and after addition of various cations to the suspending media were also performed.[227] The results appeared to indicate that, in the absence of other cations, addition of monovalent or divalent cations to the medium resulted in binding of these cations to sites on the thylakoid which were initially 'empty'. This indicates that the high fluorescing conformational state of thylakoids is brought about by different conditions if the cation binding sites investigated by these authors control fluorescence. It appears that in the absence of added cations, the high yield is due to 'empty sites' whereas in the presence of 5 mM Mn^{2+}, Ca^{2+} or Mg^{2+}, the high fluorescence results from occupation of the sites by divalent cations. When monovalent cations are bound, the fluorescence yield of chloroplasts is correspondingly low.

Such a mechanism has both practical and theoretical weaknesses however. As the authors themselves point out,[227] it offers no explanation for the effects of high concentrations of monovalent cations on fluorescence yield. There are indications that such cation binding sites contain negatively charged groups. Gross and Hess found that each site bound divalent, or two monovalent cations, indicating that electrostatic interactions between cations and binding sites are involved.[227] Berg et al[228] have argued that negatively charged carboxyl groups are involved in stacking phenomena. The stacking of thylakoids has been shown to involve a similar cation dependency to fluorescence of broken chloroplasts (see Section 4.1.2). If each cation binding site contains two negative charges, then it is clearly not possible to isolate chloroplasts (for atomic absorption analysis) which have 'empty' sites, since this would contravene the law of electroneutrality. An alternative explanation for these results will be offered in Section 4.3.

A more serious criticism is whether the type of analysis applied by

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A more serious criticism is whether the type of analysis applied by

Gross and Hess[227] is applicable to the interaction of cations with negatively charged thylakoid membranes. There are several implied assumptions in binding analys s derived from Dixon plots. One such assumption is that the effective concentration of cations at the surface of the membrane is equal to the mean cation concentration in the bulk solution. Another is that the binding of a cation to one site does not affect subsequent binding of cations to other sites (i.e. independent sites).

In this section, the effects of cations on chloroplast fluorescence havebeen re-investigated. It will be shown that the mechanism of cation induced fluorescence yield changes appear to reflect electrostatic interactions between cations and the thylakoid membrane, and only in special cases does specific binding appear to be involved. Application of simple double layer theory[229] which does not include the assumptions inherent in the analysis of Gross and Hess[225] appears to explain the results better than a mechanism involving specific cation binding to the membrane.

4.2. Results

4.2.1. Effects of ionophores on kinetics of fluorescence yield changes in broken chloroplasts

In Section 3.3, two models were presented to account for the increase in chlorophyll a fluorescence observed on adding divalent (5 mM) or monovalent cations (100 mM) to broken chloroplasts initially suspended only in low levels of monovalent cations. The models differed in the proposed location of the cation sensitive sites controlling fluorescence yield. Model I proposed that 5 mM divalent or 100 mM monovalent cations interacted with fixed negative charges on the inner side of the thylakoid membrane to induce conformational changes which increased fluorescence yield. The high energy state quenching of fluorescence (in the absence of high levels of cofactors such as DAD) was proposed to be due to displacement of these

cations from internally located sites by protons taken up in the light.

The second model also included the possibility that negative sites on the external face of the thylakoid membrane were responsible for cation induced fluorescence yield changes. In this model, 5 mM divalent or 100 mM monovalent cations acted at the externally located sites to maintain the thylakoid in a high fluorescing state. Light driven protonation of the inner side of the membrane was thought to bring about fluorescence quenching. This process, however, was visualised not to affect the binding of cations to the externally located sites.

Model I (first proposed by Barber and Telfer[194] and Krause[196]) was proposed partly to account for observations made with broken chloroplasts suspended in media containing only low levels of monovalent cations. The initial fluorescence yield of such chloroplasts was low. Illumination of broken chloroplasts under these conditions (Figure 4.1a) did not induce slow changes in chlorophyll fluorescence even though such membranes develop and maintain significant pH gradients with respect to the suspending medium (see Section 3.2.4). Addition of 100 mM K^+ (or 5 mM divalent cations) to this type of illuminated chloroplasts did not result in the normal rise in fluorescence seen in DCMU poisoned or uncoupled thylakoids. The result is particularly consistent with Model I, where light-induced proton uptake would tend to expel cations from the thylakoid interior and thus prevent the entry of cations added to the medium in the presence of the high energy state. The subsequent addition of an uncoupler (Figure 4.1a) is required to observe the cation induced fluorescence increase under these conditions. An interesting feature of this rise in fluorescence is that the kinetics are markedly faster than those observed if the high energy state is dissipated by addition of an uncoupler before the addition of 100 mM KCl (Figure 3.1b) to the medium. Careful study of this effect indicates that the results are, in fact, more consistent with Model II than

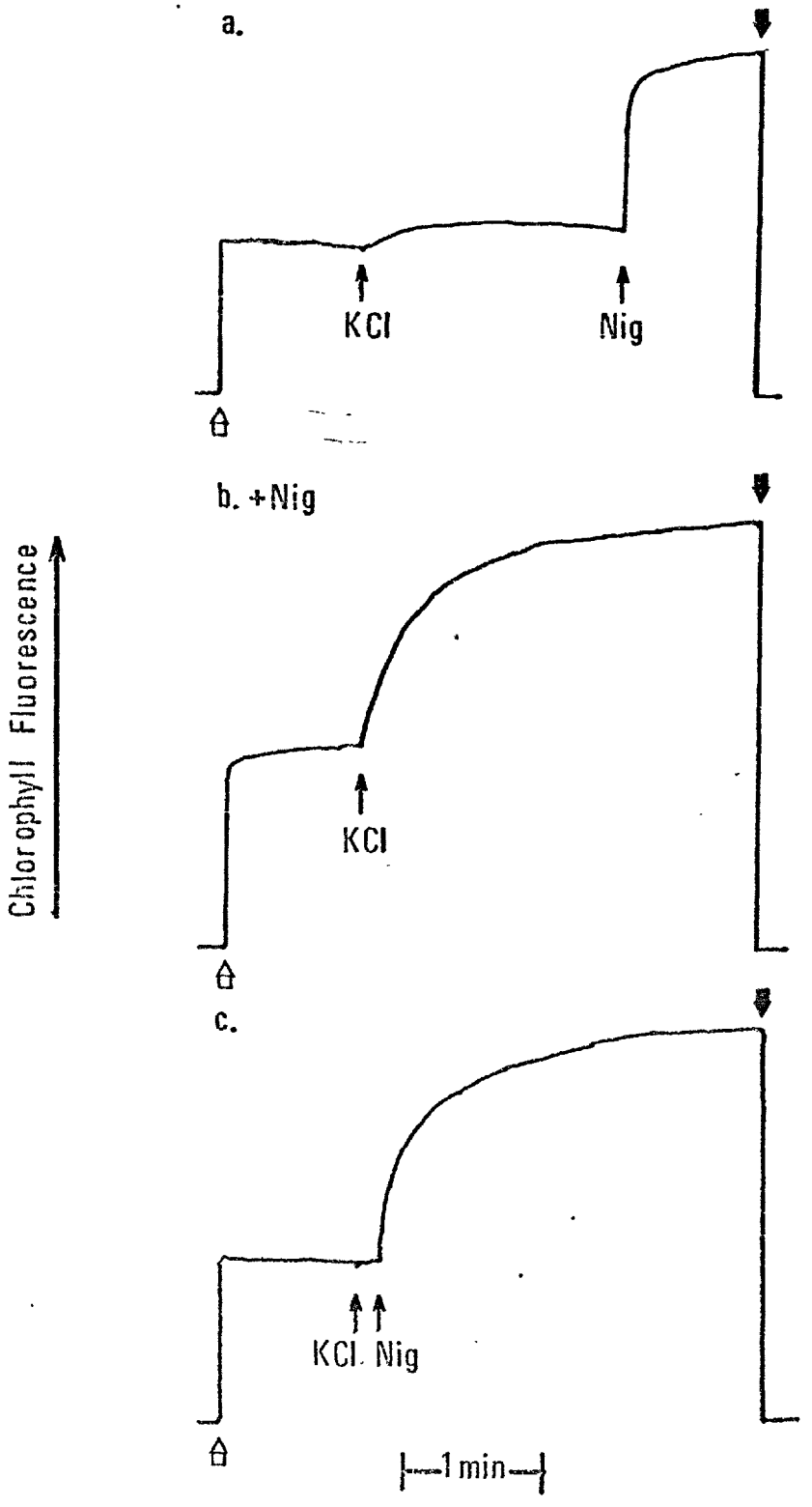


Fig. 4.1 Effect of addition of 0.1 μM nigericin at different times on the kinetics of the K^+ induced increase in chlorophyll fluorescence of broken chloroplasts suspended in assay medium. 100 mM K^+ was injected into the cuvette (containing chloroplasts at 20 μg ml^{-1}) 1 min.after illumination commenced and nigericin was added (a) 2 min. after KCl; (b) before illumination; (c) 5 sec. after KCl. Other details are given in Section 2.2.1.

Model I. This will become clear after attempting to interpret the results in terms of Model I. According to Model I, the increase in fluorescence represents the action of K^+ (when the external concentration of this cation is 100 mM) at sites on the inner surface of the thylakoid. The kinetics of the slow increase in fluorescence observed on adding 100 mM K^+ to uncoupled, broken chloroplasts (Figure 3.1b) may in principle be limited by one of three processes: (i) transport of K^+ across the thylakoid membrane, (ii) Kinetics of K^+ 'binding' to the sites controlling fluorescence, or (iii) membrane conformation changes which alter the chlorophyll fluorescence subsequent to cation 'binding'. The most likely explanation for the observed differences in the kinetics of fluorescence rise (Figure 3.1a) would be in terms of different rates of K^+ transport across the thylakoid membrane. Cation entry into the thylakoid would normally be slow in uncoupled chloroplasts (Figure 3.1b) where the cation permeability of the thylakoid would be limiting. No cation entry would occur in coupled illuminated chloroplasts (Figure 3.1a) since proton uptake would tend to drive metal cations from the intrathylakoid space. However, addition of nigericin would then speed up potassium entry of that normally seen by promoting rapid H^+/K^+ exchange across the thylakoid membrane as the ΔpH gradient collapses.

On this hypothesis, Model I predicts that increasing the permeability of the thylakoid to cations should speed up the kinetics of the slow fluorescence rise seen in the absence of a pH gradient. This was tested by studying the effects of cation specific ionophores on the rate of fluorescence increase observed on adding appropriate cations to DCMU treated broken chloroplasts suspended in low salt buffer to initially generate the low fluorescing state. Figure 4.2 shows that nigericin had no effect on the kinetics of the fluorescence rise seen on adding 100 mM KCl to DCMU poisoned broken chloroplasts. Also plotted on this figure for comparison

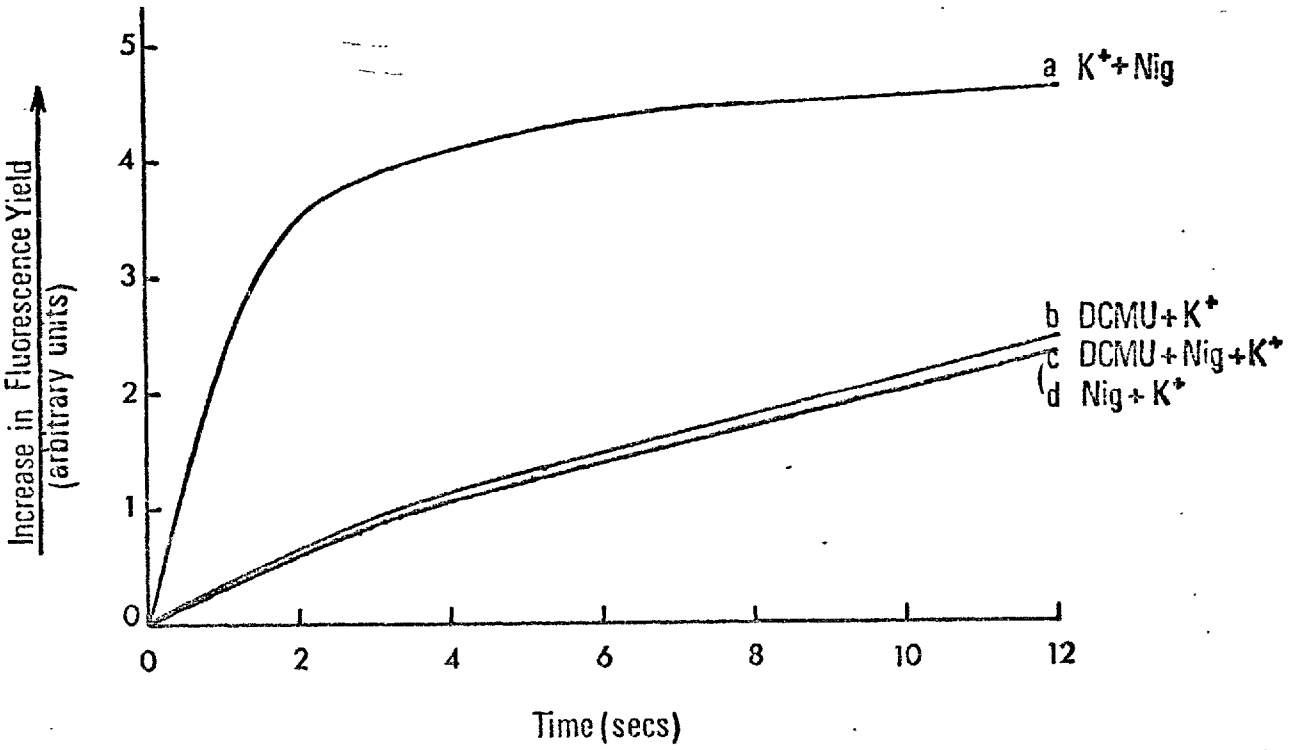


Fig. 4.2 Effect of 0.1 μM nigericin on the kinetics of the K^+ induced increase in fluorescence of broken chloroplasts suspended in assay medium. Chloroplasts were preilluminated in the presence of (a) 100 mM KCl; (b) 10 μM DCMU; (c) 0.1 μM nigericin plus 10 μM DCMU and (d) 0.1 μM nigericin for a period of 2 min. Zero time represents the addition of (a) 0.1 μM nigericin and (b),(c),(d) 100 mM KCl. In the samples preilluminated with DCMU (b),(c) nigericin had no significant effect on the kinetics of the K^+ induced fluorescence rise. In the absence of DCMU, addition of nigericin 2 min. after K^+ markedly increased these kinetics as also shown in Fig. 4.1(a). Curves (c) and (d) are superimposed. Other conditions as for Fig. 4.1.

are the faster kinetics seen on adding nigericin to chloroplasts not treated with DCMU but preilluminated in the presence of 100 mM KCl.

It was also found that ionophore A23187 was ineffective at increasing the rate of the Mg^{2+} stimulated fluorescence increase in DCMU treated broken chloroplasts in low salt buffer. Since both nigericin and A23187 facilitate cation/proton exchange across thylakoids it was possible that the rate of metal cation entry was limited by the number of exchangeable protons within the thylakoids. However, addition of gramicidin to the suspending medium had no effect on the inability of A23187 or nigericin to increase the kinetics of the Mg^{2+} or K^{+} dependent fluorescence increases. Gramicidin would be expected to stimulate re-entry of protons necessary for cation/proton exchange if this were the limiting factor.

These results indicate that the rate of the cation induced fluorescence rise in broken chloroplasts in the absence of a high energy state is not controlled by the permeability of the thylakoid.

In order to retain the hypothesis that metal cations exert their effects on fluorescence only at sites on the inner side of the membrane, it must be concluded that either the cation binding process itself or the slow subsequent membrane conformational changes limit the rate of the fluorescence rise. Either of these latter processes must somehow be speeded up in the presence of a collapsing high energy state to account for the faster kinetics of Figure 4.1a. However, there are conditions under which a collapsing pH gradient does not increase the cation induced fluorescence rise kinetics. These are shown in Figure 4.1c. Instead of allowing a few minutes between addition of 100 mM KCl and subsequent injection of nigericin, the ionophore was added a few seconds after cation addition to illuminated, coupled chloroplasts. As seen in Figure 4.1c, the fluorescence increased with slow kinetics similar to those seen in the absence of a pH gradient. It is clear that the presence of a collapsing

pH gradient does not always result in the faster fluorescence rise kinetics. The results cannot be explained by Model I but are predicted by Model II. This second model requires that cations interact with sites on the outer side of the thylakoid before high energy state quenching can be observed. Rapid kinetics of Figure 4.1a represent the relaxation of high energy state control of fluorescence as the pH gradient is dissipated by uncoupling. However, this is not seen until 100 mM KCl or 5 mM Mg^{2+} becomes associated with the fluorescence controlling sites located on the outer side of the thylakoid. This latter process must occur more slowly than the dissipation of a pH gradient. This accounts for the slow kinetics seen on adding 100 mM or 5 mM Mg^{2+} to uncoupled chloroplasts initially in the low fluorescing state. The situation is more complex when both high energy state control of fluorescence and cation effects at sites on the outer side of the thylakoid are occurring simultaneously. When 100 mM KCl and uncoupler are added together to chloroplasts in the low fluorescence state, the rate of the subsequent fluorescence rise is controlled by the action of cations at the externally located sites (slow, Figure 4.1c). However, when uncoupler is added some time after salt addition to coupled illuminated membranes, events at the external sites have been completed, and the rate of fluorescence rise is controlled by deprotonation of the internal sites (fast, Figure 4.1a). The fast and slow rise kinetics of Figure 4.1 seem to represent, therefore, two distinct processes which have inherently different rate constants, and which occur at spatially separated sites. The results are therefore consistent with Model II, where cations mainly exert their influence on chlorophyll fluorescence at negatively charged sites on the external surface of the thylakoid. This model therefore predicts that both membrane permeable and impermeable cations should induce fluorescence yield changes in isolated broken chloroplasts provided that steric factors do not interfere with interactions between such im-

permeable cations and the sites controlling fluorescence. Table 4.1 lists some cations which increased fluorescence from DCMU treated broken chloroplasts suspended in low salt buffer. It is clear that the relatively impermeable[230] choline cation was almost as effective as potassium under such conditions, but that equiosmolar sucrose had no effect. It was also found that in the absence of DCMU, the high fluorescent state created by choline addition to dark, coupled chloroplasts showed the same light induced high energy state quenching as observed with appropriate levels of metal cations in the medium. The light induced quenching observed in the presence of high concentrations of choline was inhibited on addition of uncouplers to the medium. It seems unlikely that such high energy state quenching involves displacement of choline by protons at sites on the innner side of the thylakoid as proposed by Model I. The results are well accommodated by Model II however where light dependent protonation of the inner side of the thylakoid displaces cations other than choline. In this model high concentrations of choline creates the high fluorescing state via interactions of this cation at externally located sites, and this allows the high energy state effects to be observed.

4.2.2. The role of monovalent cations in control of chlorophyll a fluorescence from broken chloroplasts suspended in cation free-media

In the previous section, evidence was presented that externally located sites on the thylakoid can control fluorescence of isolated, broken chloroplasts. In order to investigate this possibility further, the antagonistic effects of low concentrations of monovalent and divalent cations on fluorescence yield (as originally reported by Gross and Hess[172]) were studied. In all the experiments so far reported in this thesis, the suspending media contained sufficient concentrations of monovalent cations to initially create the low fluorescing state of isolated broken chloroplasts in the absence of other added cations.

TABLE 4.1.

The effect of various additions on chlorophyll fluorescence of broken chloroplasts[(a)] treated with 2 x 10^{-5} M DCMU.

Addition	Fluorescence yield (Arb. units)	Concentration for half effect ($C_{\frac{1}{2}}$) (mM)
None	30	-
0.4 M Sucrose	29	-
0.2M KCl	76	65
0.2 M Choline Cl	63	95
5 mM $MgCl_2$	73	0.8

(a) Broken chloroplasts (15 µg chlorophyll ml^{-1}) suspended in low salt buffer. Fluorescence refers to steady state yield in high intensity illumination (see Section 2.2.1.).

However, when broken chloroplasts were washed and resuspended in essentially cation-free media (containing only sucrose and 0.1-0.2 mM tris, see Section 2.1.2) the initial fluorescence yield of DCMU treated membranes was relatively high. Addition of monovalent cations up to 10 mM decreased this high yield of fluorescence to a low level, as shown in Figure 4.3. Such monovalent cation-induced fluorescence lowering was not due to high energy state effects which have been eliminated in these preparations by the addition of DCMU. This is also true for all of the cation induced fluorescence changes to be reported subsequently in this thesis. As Figure 4.3 shows, the monovalent cation-induced fluorescence lowering of broken chloroplasts suspended in a cation-free medium was remarkable unspecific. Within the limits of experimental error, all of the alkali (group Ia) metal cations were equally effective with the concentration inducing half the maximum fluorescence lowering ($C_{\frac{1}{2}}$) being 0.8 $\pm$ 0.2 mM. Table 4.2 lists the $C_{\frac{1}{2}}$'s obtained in this experiment (Expt. 1). In fact, it was generally observed that the $C_{\frac{1}{2}}$ for any particular cation varied more between different batches of chloroplasts than the $C_{\frac{1}{2}}$ measured for different alkali metal cations within any one batch of chloroplasts. Table 4.2 lists the typical values of $C_{\frac{1}{2}}$ found for K^+ induced fluorescence lowering from several experiments with broken chloroplasts suspended in cation-free media.

As shown in the earlier sections and previously by many authors[169,172,175] chlorophyll fluorescence can be restored from the low yield (seen in the presence of 10 mM monovalent cations) to the high yield by further increasing the monovalent cation concentration of the suspending medium. For broken chloroplasts suspended in a cation-free medium, a complete concentration curve for monovalent cations shows first a quenching of fluorescence (0-10 mM) and then an increase in emission intensity (10-100 mM monovalent cations). This is shown for several different monovalent cations in

Fig. 4.3 Effect of the monovalent alkali metal cations on the chlorophyll fluorescence yield of broken chloroplasts washed and resuspended in cation-free medium (0.1 M sorbitol plus approximately 0.1-0.2 mM tris, pH 7.0). Chloroplasts were preincubated at a chloroplast concentration of 8 μg ml^{-1} with 13.3 μM DCMU and the appropriate concentration of LiCl (solid squares), NaCl (open squares), KCl (solid circles), RbCl (open circles), or CsCl (triangles) for 7.5 min. before noting the final steady-state fluorescence level observed in high intensity illumination (see Section 2.2.1).

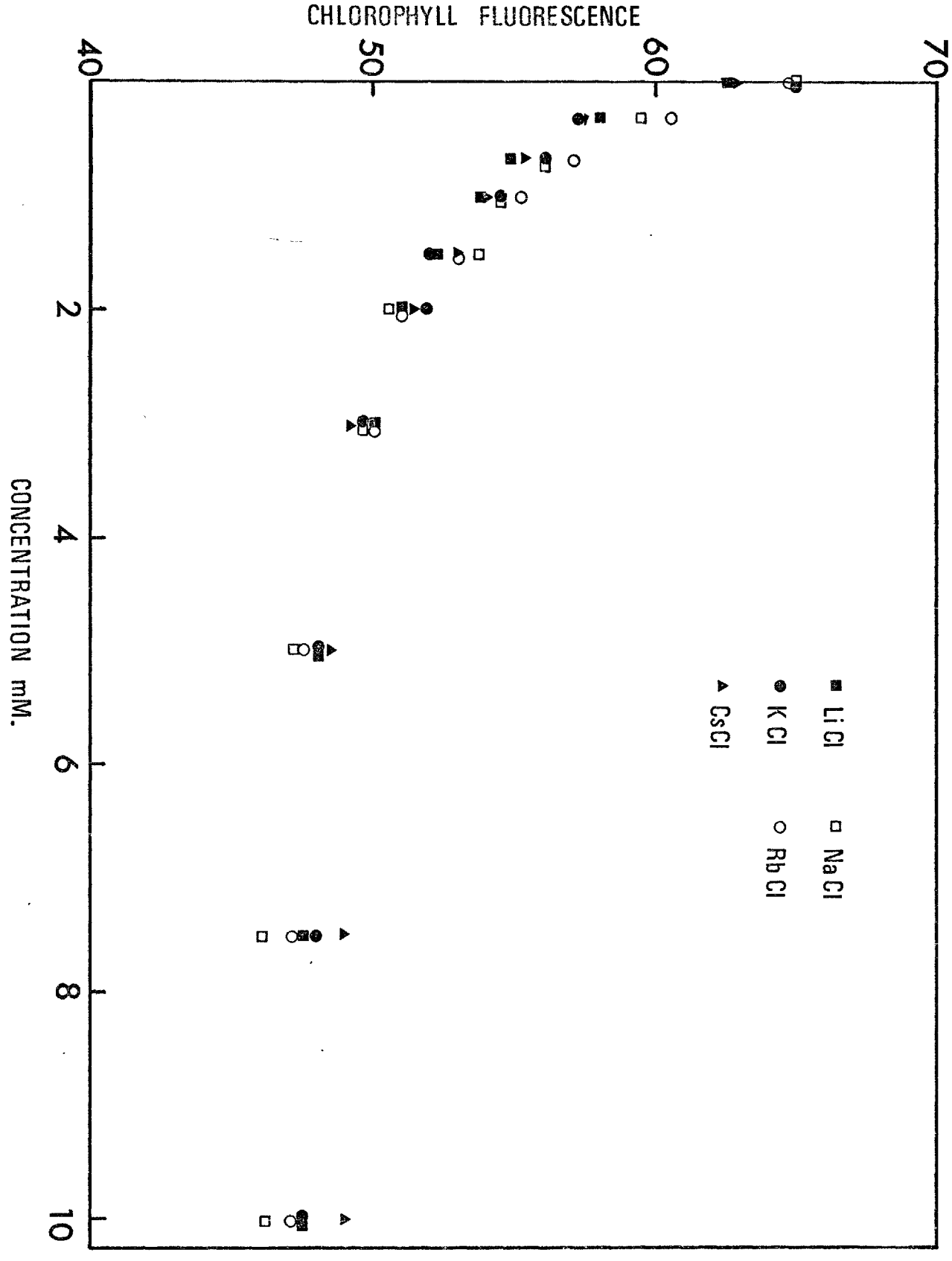

CHLOROPHYLL FLUORESCENCE
40
50
60
70
CONCENTRATION mM.
2
4
6
8
10
Li Cl
Na Cl
K Cl
Rb Cl
CsCl

TABLE 4.2.

Relative effectiveness of different monovalent cations on chlorophyll fluorescence of DCMU treated broken chloroplasts initially suspened in a cation-free medium[a].

Experiment	Salt	$C_{\frac{1}{2}}$ of cation induced fluorescence change (mM)	
		Decrease	Increase
1	LiCl	0.7 ± 0.2	-
	NaCl	0.8 "	-
	KCl	0.7 "	-
	RbCl	0.9 "	-
	CsCl	0.9 "	-
2	KCl	1.3 ± 0.3	45 ± 5
	Choline Cl	1.6 "	65 "
	NH_4 Cl	1.5 "	approx. 30[b]
	HEPES/tris	20 ± 4	No effect
3	tris Cl	2.5 ± 5	approx. 50[b]
4	KNO_3	1.2 ± 0.3	35 ± 5
	Lysine Cl	0.8 ± 0.2	65 "
5	KNO_3	1.3 ± 0.3	41 ± 5
	$TlNO_3$	0.8 ± 0.2	No effect[b]

(a) Chloroplasts washed and resuspended in a cation-free medium containing 1.3×10^{-5} M DCMU. Final concentration of chloroplasts was 8 - 10 µg chlorophyll ml^{-1}. For other details see Sections 2.1.2 and 2.2.1.

(b) Obscured by irreversible fluorescence lowering (see text).

Figures 4.4, 4.5 and Table 4.2.

It is clear that the effectiveness of monovalent cations in decreasing fluorescence at low concentrations (10 mM) and increasing this emission at high concentrations, is not restricted to monovalent, metal cations. Both types of fluorescence change were induced by small organic monovalent cations such as NH_4^+, and larger bulkier groups such as choline, tris and lysine. There seems to be very little structural requirement necessary in the nature of the monovalent cation in order to be effective at changing the fluorescence yield of chloroplasts. This seems to emphasise that the cation sensitive sites controlling fluorescence must be highly exposed (Model II, Figure 3.21) and not buried within the thylakoid (Model I).

Although the structural properties appear to be relatively unimportant, there is a strict requirement that the cation be monovalent to observe the fluorescence effects of Figure 4.4. This can be seen by comparing the effects of HEPES (Figure 4.5b) and lysine (Figure 4.4c). At the pH 7.0 (used in this experiment) the ε-amino group of lysine is protonated. Although at this pH lysine also contains a Zwitterionic group, the overall net positive charge on the molecules is one, and thus fulfills the condition of monovalency. On the other hand, HEPES at pH 7.0 is Zwitterionic but electrically neutral. As seen in Figures 4.4 and 4.5, HEPES was apparently much less effective than the monovalent lysine cation, and brought about fluorescence lowering with an apparent $C_{\frac{1}{2}}$ tenfold higher than monovalent cations. However, even this apparent fluorescence quenching by HEPES could be ascribed completely to the tris cation which was added (as the tris base) to the HEPES stock solution in order to bring the pH to 7. This tris cation concentration was tenfold less than the final HEPES concentration. As shown in Figure 4.5a and Table 4.1, tris (almost fully protonated at pH 7.0 and hence monovalent) was as effective as potassium and other monovalent cations in bringing about fluorescence quenching of

Fig. 4.4 Dependence of chlorophyll fluorescence from broken chloroplasts washed and resuspended in cation-free medium on the concentration of monovalent cations added to the medium. Conditions as for Fig. 4.3.

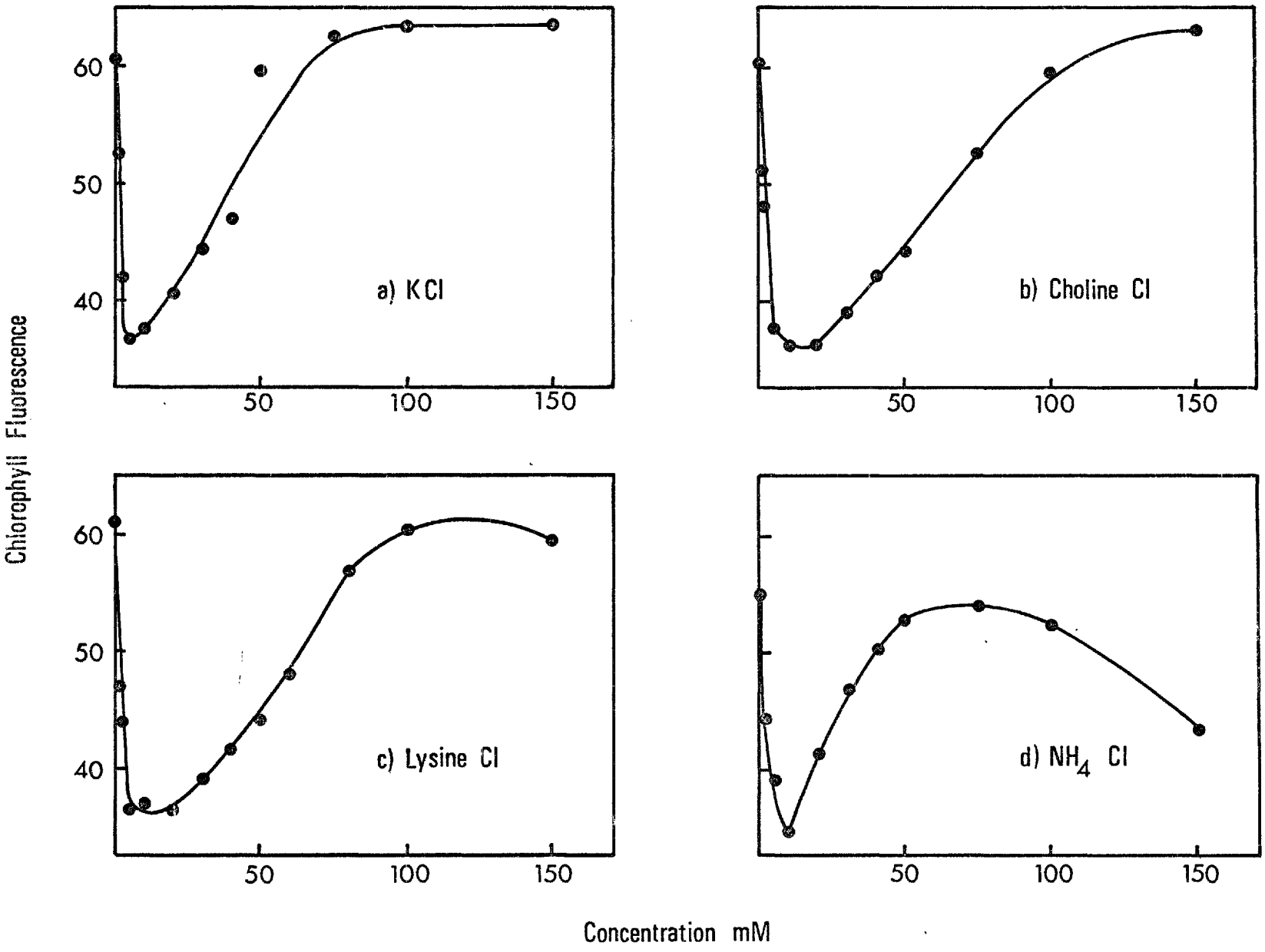
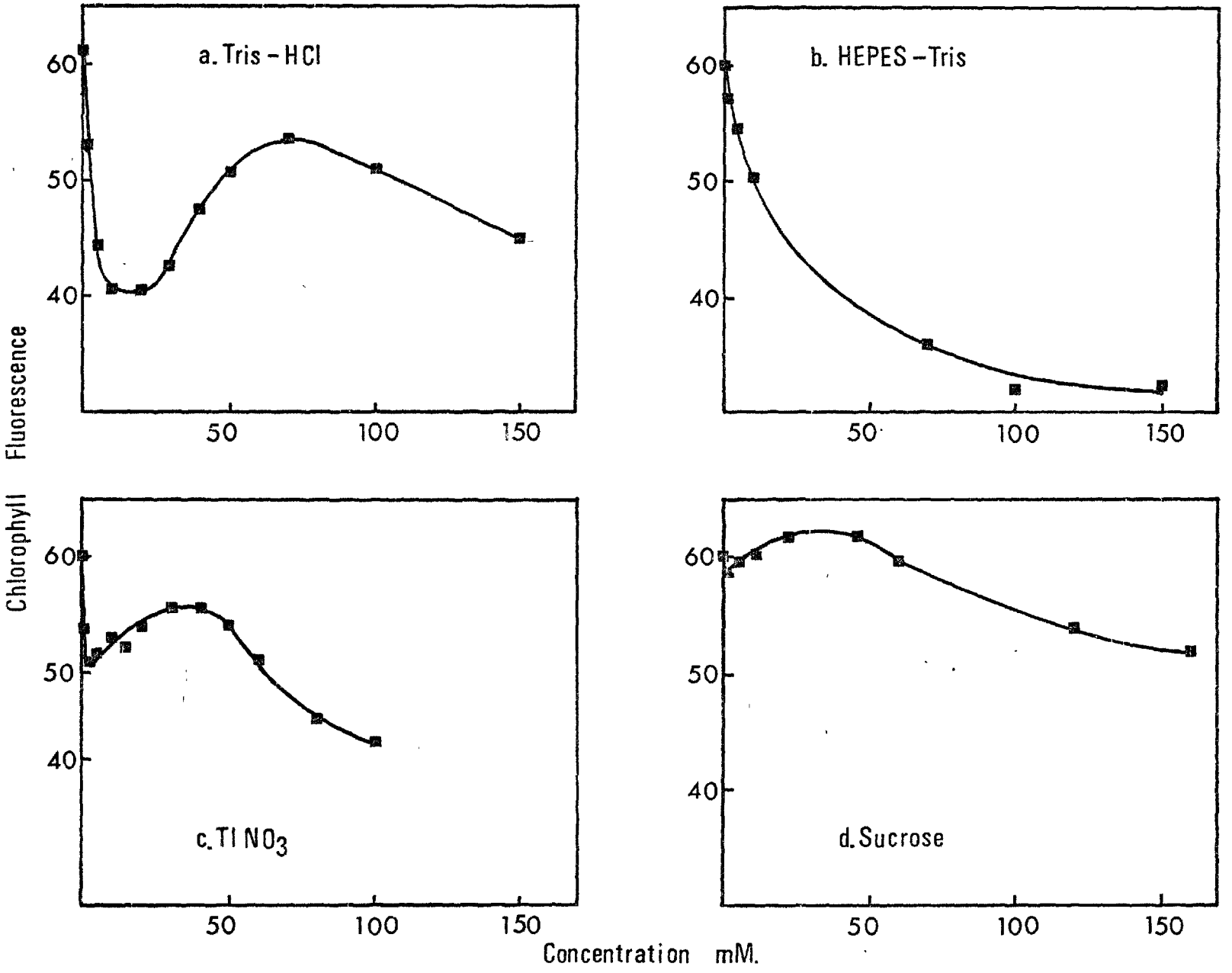

Chlorophyll Fluorescence
60
50
40
a) KCl
50
100
150
b) Choline Cl
c) Lysine Cl
d) NH_4 Cl
Concentration mM

Fig. 4.5 Dependence of chlorophyll fluorescence from broken chloroplasts washed and resuspended in cation-free medium on the concentration of monovalent cations or sucrose added to the medium. Conditions as for Fig. 4.3.


a. Tris - HCl
b. HEPES - Tris
c. Tl NO3
d. Sucrose
Chlorophyll Fluorescence
Concentration mM.
60
50
40
50
100
150

chloroplasts in cation-free media. It appears therefore that the fluorescence lowering brought about HEPES/tris was due entirely to the smaller levels of tris cation present. The electrically neutral HEPES Zwitterion seems to be ineffective at inducing the characteristic fluorescence lowering and subsequent increase at high concentrations, and in this respect resembles uncharged molecules such as sucrose (Figure 4.5d). It may be asked why the initial fluorescence yield of broken chloroplasts suspended in cation-free media is high when in fact this medium contains tris cation added as the base to bring the medium to pH 7? The concentration of tris employed for this purpose, however, was only 0.1-0.2 mM (final concentration) and is thus too low to effect appreciable fluorescence lowering of broken chloroplasts.

The fluorescence yield changes so far discussed are all largely reversible between the low and high yield of broken chloroplasts on varying the monovalent cation constitution of the medium. For ease of reference, such fluorescence changes induced by monovalent cations will be termed reversible fluorescence changes. Such reversible fluorescence changes are completely prevented by the presence of 5 mM divalent cations such as Mg^{2+} in the medium. 5 mM Mg^{2+} maintained chloroplasts at the initial high fluorescing yield even on addition of 10 mM monovalent cations to the suspending media.

A notable feature of reversible fluorescence yield changes induced by monovalent cations is the almost complete lack of specificity between cations in terms of ionic size and structure, but the absolute requirement for the cation to be monovalent. These results suggests that monovalent cations exert their effects on chloroplast fluorescence via electrostatic interactions with exposed, negative charges on the outer side of the thylakoid membrane, rather than by specific binding to ligands on the membrane. Such specific binding, if it occurred, might be expected to

be highly dependent on the ionic size and coordination number of the monovalent cations.[231,232]

However, other effects of monovalent cations at very high concentrations may well involve specific binding to the membrane. Figures 4.4. and 4.5 show that certain cations such as tris and NH_4^+ were able to bring about the reversible chloroplast fluorescence quenching at low concentrations and subsequent increase at high cation concentrations as discussed above. However, on further increasing the levels of tris or NH_4^+ in the suspending medium, the fluorescence of broken chloroplasts was again decreased from the high yield to a lower level. Apart from the concentration dependence, the fluorescence decrease brought about by high levels of monovalent cations could be distinguished from that seen at low concentrations (0-10 mM) in two other respects. The high-cation fluorescence quenching was not prevented by the presence of 5 mM Mg^{2+} in the medium and was markedly dependent on the nature of the monovalent cation used. This type of fluorescence decline will be termed irreversible fluorescence quenching. Irreversible chloroplast fluorescence quenching was rarely observed with K^+ or choline (Figure 4.4) but was clearly seen when primary amines such as lysine, tris and ammonium cations were used. Primary amines at high concentrations are known to induce other effects on chloroplasts such as an inhibition of oxygen evolution.[141,144,233] It seems likely, therefore, that irreversible fluorescence quenching of broken chloroplasts may be associated with specific interactions of certain monovalent cations with the thylakoid. Thallium, a monovalent metal cation suggested by Williams[231] as a possible analogue for K^+, appeared to be particularly effective at irreversibly quenching chloroplast fluorescence. However, this cation did induce reversible fluorescence changes in broken chloroplasts when lower concentrations of Tl^+ were used.

4.2.3. Antagonistic effects of monovalent and divalent cations on chloroplast fluorescence

Figure 4.6 C shows that the fluorescence lowering on adding 10 mM K^+ to broken chloroplasts washed and suspended in a cation-free medium was fully reversed back to the high yield on injecting 10 mM Mg^{2+} into the reaction medium. This experiment demonstrates the antagonistic effects of monovalent and divalent cations at low concentrations (2-10 mM) on chloroplast fluorescence.[172]

The question now arises in the search for a coherent explanation of cation induced fluorescence changes, as to what causes the high initial yield of such chloroplasts washed and suspended in a cation-free medium? The first clue to the answer to this question came with observations that the initial yield of fluorescence of broken chloroplasts suspended in cation-free media was somewhat variable between different chloroplast preparations. The initial yield of fluorescence was often found to be at different intermediate levels between the minimum (seen in the presence of 10 mM monovalent cations) and maximum (10 mM divalent cations) observable yields. It was then found that the initial yield could be further reduced by washing membranes in media containing a few mM monovalent cations. This is shown in Figure 4.6. Broken chloroplasts were subjected to a single wash in cation-free medium with 1mM KCl additionally present. The chloroplasts were collected by centrifugation and resuspended in cation-free medium without further additions (except for DCMU). Another sample of chloroplasts from the same original batch was subjected to identical treatment except 1 mM K^+ was omitted from the washing medium. It can be seen that these latter chloroplasts exhibited a high initial fluorescence which was reduced by KCl (10 mM) and restored to the original level by Mg^{2+} (10 mM) added to the medium (Figure 4.6C). However, those chloroplasts subjected to washing in 1 mM KCl showed a depressed initial fluorescence

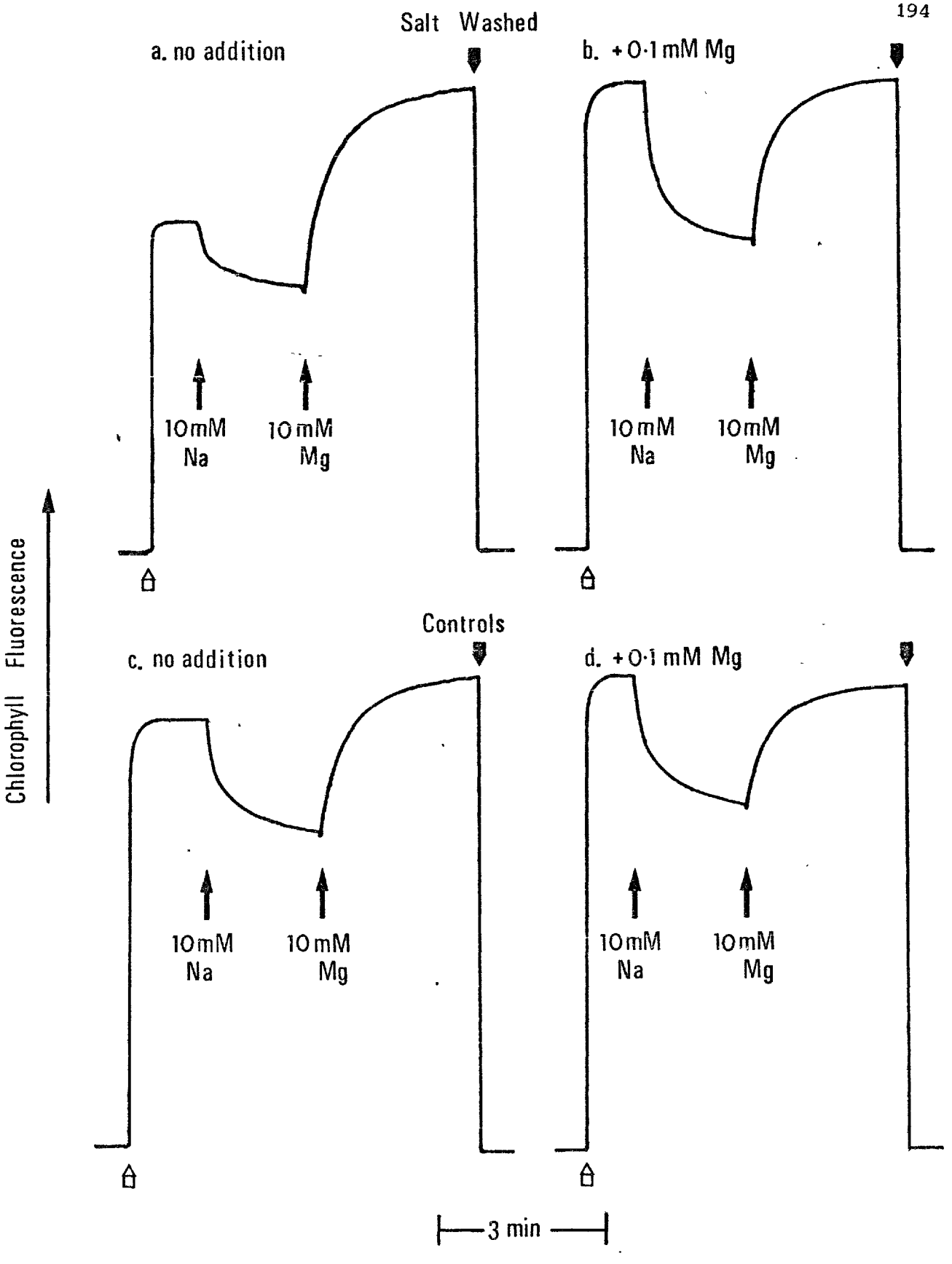


Fig. 4.6 Antagonistic effects of monovalent and divalent cations on fluorescence from broken chloroplasts washed and resuspended in cation-free medium containing 10 μM DCMU. Washing medium (but not the final resuspending medium) contained additionally 1 mM KCl for samples (a) and (b). Samples (a),(c) no further additions; (b), (d) plus 100μM $MgCl_2$. See Section 2.2.1 for other experimental details.

even though the suspending medium contained no added monovalent cations (Figure 4.6c). The levels of fluorescence seen in the subsequent presence of 10 mM K^+ and 10 mM K^+ plus 10 mM Mg^{2+} were little altered from the levels observed in Figure 4.6a. Furthermore, the depressed initial fluorescence level of KCl-washed chloroplasts in the absence of other added cations could be restored to the maximum yield by adding extremely low levels (100 μM) of divalent cations. Normal quenching from this restored yield was seen on adding 10 mM K^+ to the medium (although the final extent of quenching was slightly reduced). Addition of 100 μM $MgCl_2$ had little effect on the initial fluorescence of those chloroplasts washed in cation-free medium (-KCl), but again, did slightly inhibit the extent of subsequent K^+ induced fluorescence lowering. The results clearly show the antagonistic effects of monovalent and divalent cations on fluorescence of chloroplasts suspended in media of very low ionic strength.

These observations suggest that the high fluorescence yield seen when membranes are not washed in monovalent cations may be due to residual divalent cations already associated with the thylakoids. In Section 3.2.2 evidence was obtained that broken chloroplasts retain sufficient divalent cations to enable ionophore A23187 to exert its uncoupling action without the further need to add divalent cations.

The fluoresence lowering induced by 10 mM monovalent cations added to chloroplasts in cation-free media may be due to competitive displacement of residual Mg^{2+} from the sites controlling fluorescence. If this is so, replacement of the anion in the monovalent salt by EDTA should facilitate any displacement of divalent by monovalent cations at the membrane surface. As shown in Figure 4.7, sodium EDTA ($C_{\frac{1}{2}} \sim 0.1$ mN) was more effective than sodium chloride ($C_{\frac{1}{2}} \sim 0.9$ mN) in decreasing fluorescence of broken chloroplasts suspended in a cation-free medium when the two salts were compared at equivalent sodium concentrations. No such increased effectiveness was observed when sodium nitrate or sulphate were tested.

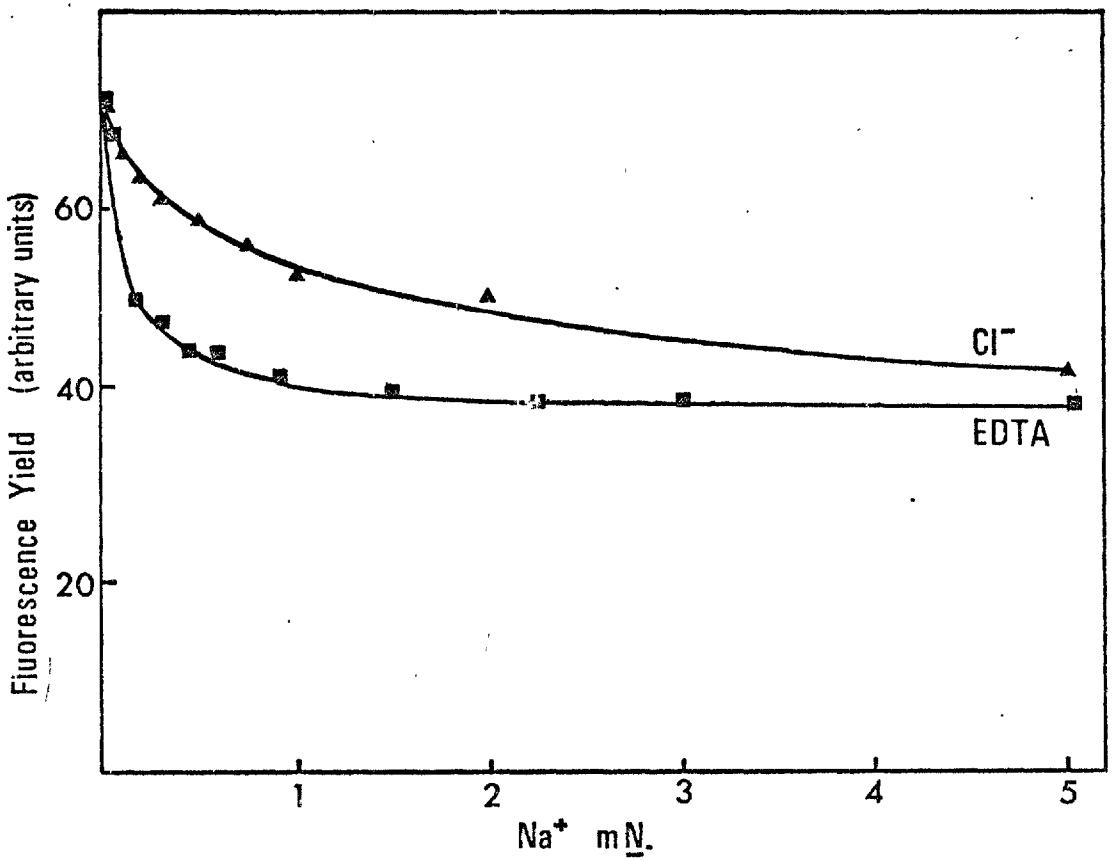


Fig. 4.7 Comparison of the relative effectiveness of the chloride and EDTA salts of sodium on the monovalent cation induced decrease in fluorescence yield of broken chloroplasts washed and resuspended in cation-free medium. Conditions as for Fig. 4.3.

The results suggest that the antagonism exerted between monovalent and divalent cations on chloroplast fluorescence may be due to competition between these cations for the thylakoid negative sites proposed to control fluorescence. This suggestion is supported by results shown in Figure 4.8. In this experiment, broken chloroplasts were washed in 0.5 mM EDTA, treated with DCMU, and resuspended in media containing different concentrations of KCl. As the vertical axis of Figure 4.8a shows, the initial fluorescence yield of such chloroplasts is depressed even in the absence of added KCl. This initial fluorescence is further decreased to the low level in media containing 10 mM K^+. Addition of Mg^{2+} (horizontal axis, Figure 4.8a) increases fluorescence to the high level, but the $C_{\frac{1}{2}}$ for this divalent cation effect is progressively increased in media containing increased levels of monovalent cation. The double reciprocal plot (Figure 4.8b) of the data in Figure 4.8a, suggests that K^+ competitively inhibits the Mg^{2+} induced increase of fluorescence from the low yield.

In the presence of 10 mM KCl, the $C_{\frac{1}{2}}$ for Mg^{2+} induced fluorescence rise of broken, EDTA-washed chloroplasts was 0.4 mM, in agreement with earlier reports.[157,170] However, in the absence of any monovalent cations (apart from low levels of tris in the medium), the $C_{\frac{1}{2}}$ for the Mg^{2+} induced fluorescence rise was much lower and of the order 50 μM. This observation may explain why thylakoids cannot be depleted of divalent cations by simply washing the membranes in cation-free media. Because thylakoids apparently bear a net negative charge,[228,234] these membranes would be expected to retain divalent cations throughout any such washing procedure in order to maintain overall electroneutrality at the membrane surface. These levels of retained divalent cations are apparently sufficient to create the high fluorescing state of chloroplasts in media containing no other cations. Introduction of sufficient monovalent cations into the washing or suspending media is apparently required to displace these

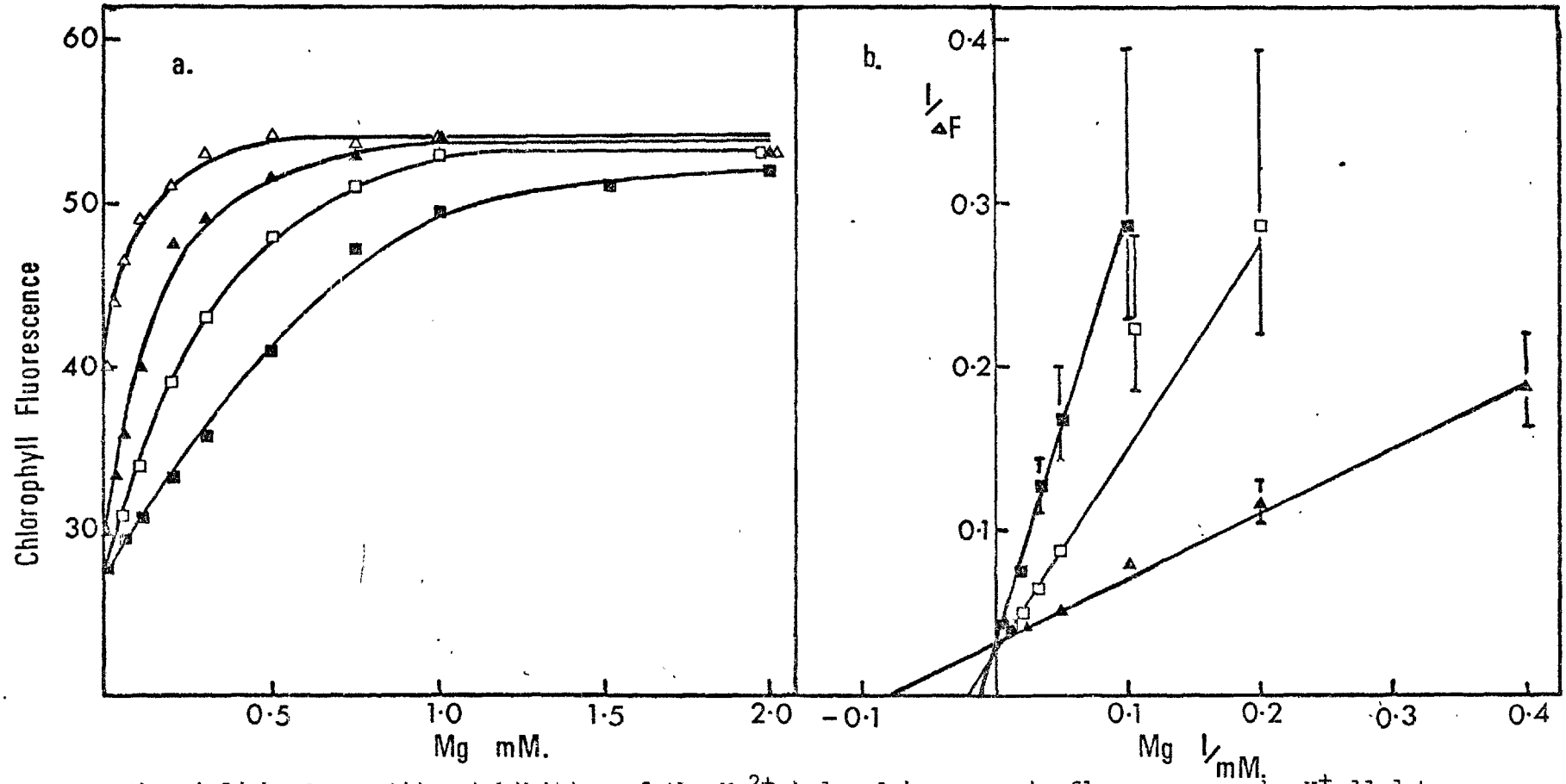


Fig. 4.8(a) Competitive inhibition of the Mg^{2+} induced increase in fluorescence by K^{+}added to broken chloroplasts initially washed in EDTA and resuspended in cation-free medium. Final medium contained 10 μM DCMU and: open triangles, no further additions; solid triangles, 1 mM KCl; open squares, 3 mM KCl; solid squares, 10 mM KCl. (b) Double reciprocal plot of the data in (a). Other conditions as for Fig. 4.3.

retained divalent cations, and this results in the low fluorescing state.

4.2.4. Specificity of divalent cations on chlorophyll fluorescence yield changes in broken chloroplasts

Monovalent cations were shown to exhibit broad specifity in their ability to decrease the initial high chlorophyll a fluorescence yield of broken chloroplasts suspended in a cation-free medium. Divalent cations also show little difference in their relative ability to restore this decreased fluorescence to the high level. Figure 4.9 and Table 4.3 shows that all of the alkaline earth (Group IIa) metal cations are effective at much lower concentrations than those previously reported[157 170] when chloroplasts were washed in EDTA and resuspended in a cation-free medium to create the initial low fluorescing state. It was generally observed that Mg^{2+} was slightly less effective than the other alkaline earth cations under these conditions (Figure 4.10 and Table 4.3). However, this small difference in relative effectiveness of divalent cations is insignificant in comparison to the much higher levels of monovalent cations required to create the high fluorescing state.

Figure 4.11 and Table 4.3 show that the ability to increase the fluorescence yield of chloroplasts under these conditions is not confined to Group IIa divalent metal cations. Both Mn^{2+} and Co^{2+} were also observed to create the high fluorescing state at similarly low concentrations (Table 4.3). For the sake of convenience this general divalent cation induced increase in fluorescence from the low yield will also be termed 'reversible fluorescence changes'. Figure 4.11 shows, however, that like some monovalent cations, certain divalent cations at high concentrations induce a decline in chlorophyll fluorescence (termed 'irreversible quenching'). The magnitude of this irreversible quenching seems to depend on the cation used. Zn^{2+} (Figure 4.11), Cd^{2+} and UO_2^{2+} (data not shown) induced a drastic

Fig. 4.9 Increase in the chlorophyll fluorescence from EDTA washed broken chloroplasts induced by the presence of divalent alkaline earth cations in an otherwise cation free suspending medium. Chloroplast concentration was 10 $\mu g\ ml^{-1}$, and all samples contained 13.3 μM DCMU. Other experimental conditions as for Fig. 4.3.

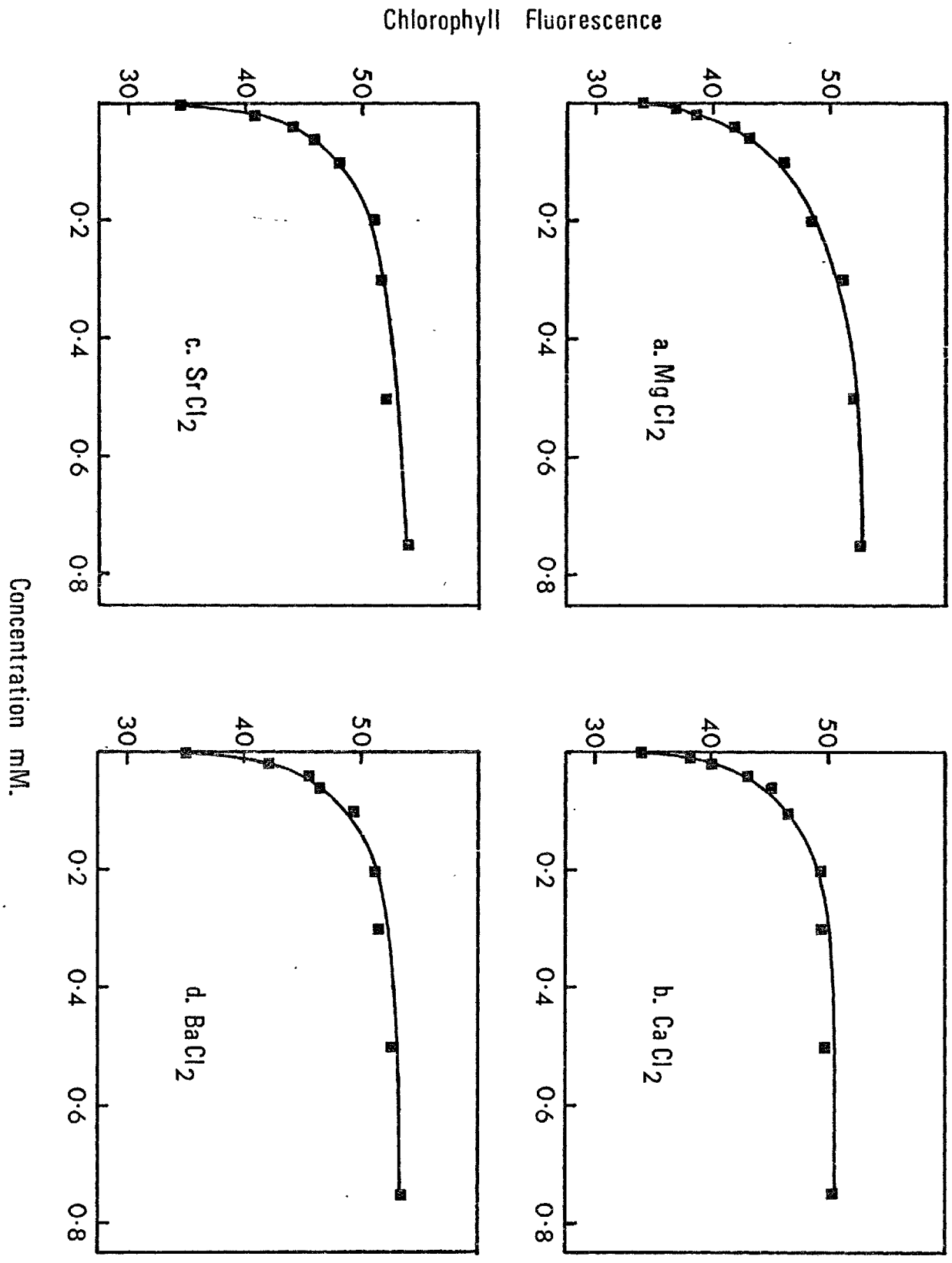

Chlorophyll Fluorescence
a. $MgCl_2$
b. $CaCl_2$
c. $SrCl_2$
d. $BaCl_2$
30
40
50
0·2
0·4
0·6
0·8
Concentration mM.

TABLE 4.3.

Relative effectiveness of different divalent cations at increasing chlorophyll fluorescence of EDTA washed broken chloroplasts suspended in a cation-free medium[(a)].

Experiment	Salt	$C_{\frac{1}{2}}$ for cation induced fluorescence increase (μM)
1	$MgCl_2$	62 ± 5
	$CaCl_2$	44 "
	$SrCl_2$	42 "
	$BaCl_2$	36 "
2	$MgCl_2$	70 ± 10
	$MnCl_2$	30 "
	$CoCl_2$	approx. 10[(b)]
	$ZnCl_2$	No effect[(b)]
3	$MgCl_2$	50 ± 5
	Lysyl-L-lysine	170 ± 20

(a) Chloroplasts 8 - 10 μg ml^{-1} treated with 1.3 x 10^{-5} M DCMU.

(b) Obscured by irreversible fluorescence quenching (see text).

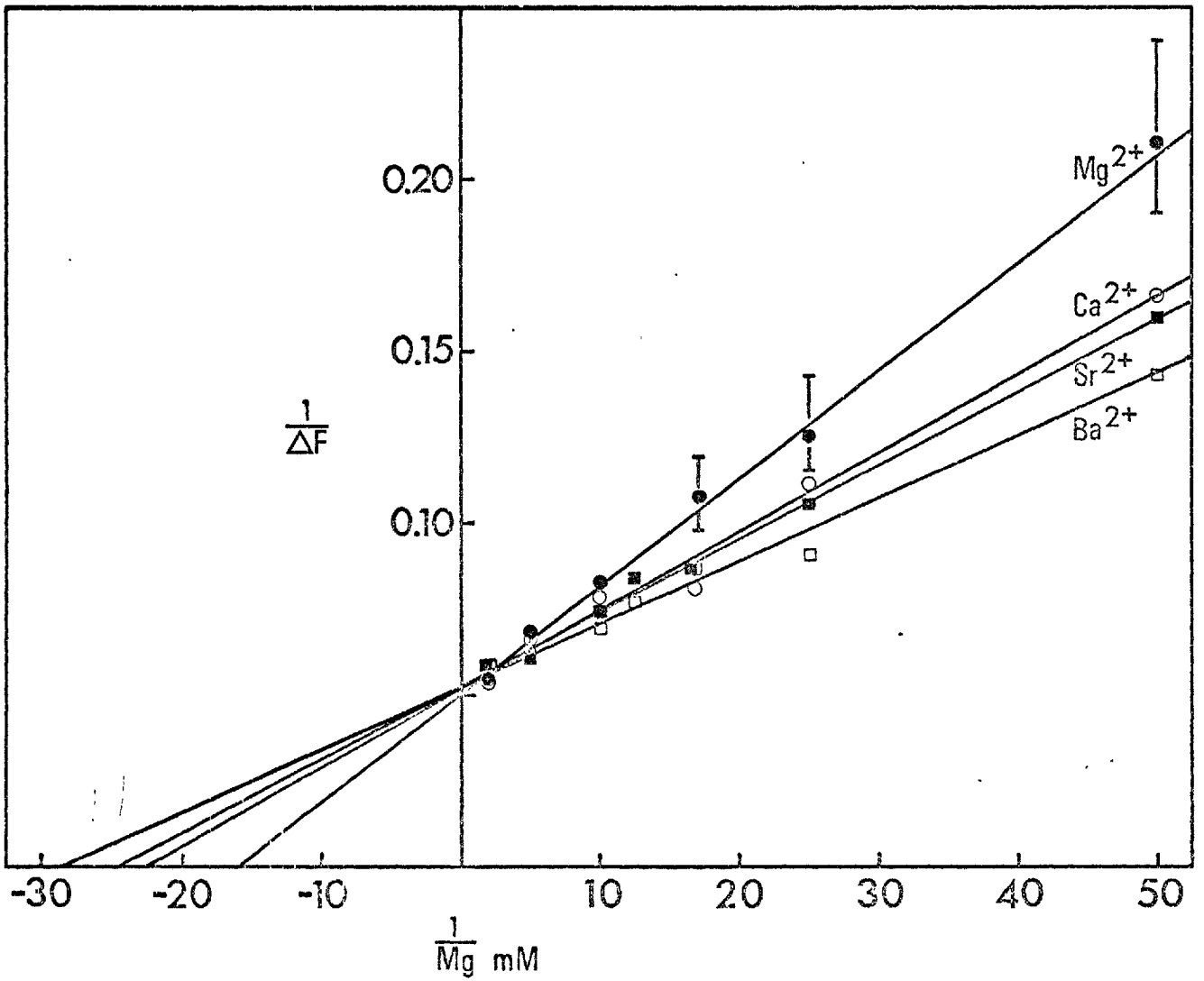


Fig. 4.10 Double reciprocal plot of the data shown in Fig. 4.9.
ΔF is the increase in fluorescence (arbitrary units) induced by divalent cation in comparison to the control sample containing no added cations.

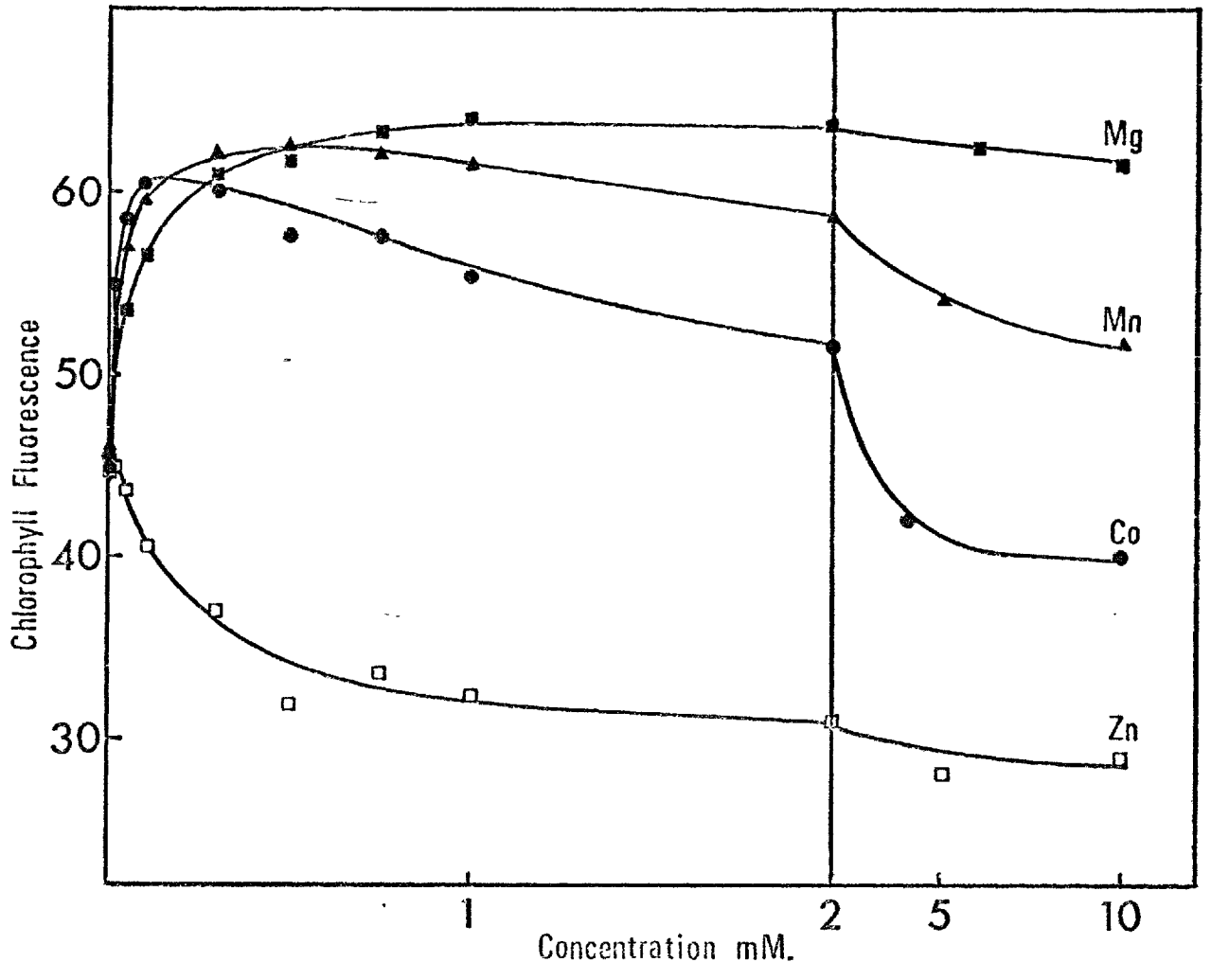


Fig. 4.11 Dependence of fluorescence from EDTA-washed broken chloroplasts on the divalent cation content of an otherwise cation free chloroplast suspending medium. Other conditions as for Fig. 4.9.

quenching of fluorescence which probably totally obscured any reversible fluorescence increase these cations might also have induced.

The irreversible fluorescence decline induced by divalent cations appears to be cation specific and may therefore be due to specific binding of these cations to the thylakoid. However, the reversible fluorescence changes (increase from the low yield) brought about by divalent cations exhibits relatively little specificity on the basis of ionic size or coordination number (c.f. Ca^{2+}, Mg^{2+}, Ref. 231, 232). This latter type of fluorescence change may be better explained by electrostatic interactions between divalent cations and the negatively charged thylakoid as discussed in Section 4.3.

To explore this point further, it was of interest to investigate the action of the dipeptide lysyl-L-lysine on the fluorescence yield of isolated broken chloroplasts. The monomer lysine has previously been shown to act as a typical monovalent cation, decreasing chloroplast fluorescence at low concentrations (0-10 mM) and restoring fluorescence to the maximum yield at higher lysine concentrations (20-100 mM). However, the dimer lysyl-L-lysine was not observed at any concentration to decrease the chlorophyll a fluorescence of DCMU treated chloroplasts washed and suspended in a cation-free medium. Moreover, this dipeptide acted as a typical divalent cation as shown in Figure 4.12. Lysyl-L-lysine was observed to increase fluorescence of EDTA washed broken chloroplasts in a manner which was very similar to divalent cations such as Mg^{2+}. The observed $C_{\frac{1}{2}}$ for lysyl-L-lysine induced fluorescence was only threefold higher than that for Mg^{2+} (Table 4.3, c.f. lysine where $C_{\frac{1}{2}}$ was approximately 1,000-fold that of Mg^{2+}). Thus, although the distance between the two ε-amino groups of a lysyl-L-lysine molecule can be relatively large (depending on the molecular conformation) this species appears to act only as a divalent cation.

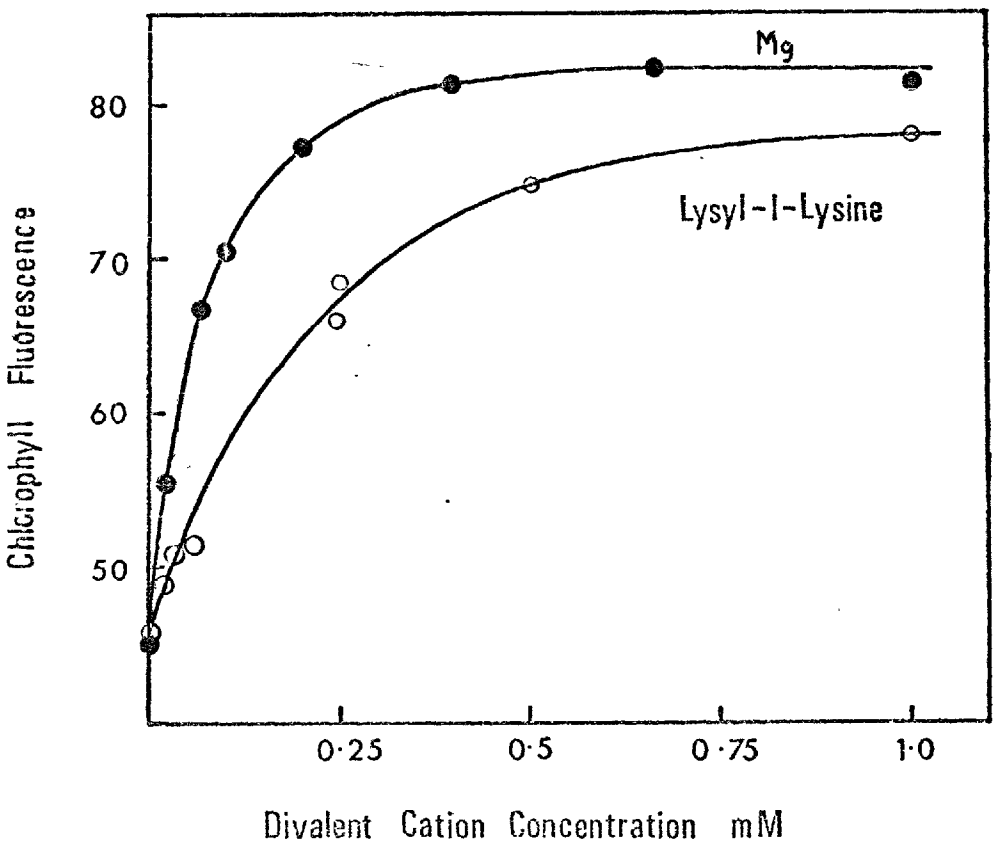


Fig. 4.12 Effect of the divalent cations Mg^{2+} and lysyl-L-lysine on chlorophyll fluorescence from EDTA washed broken chloroplasts suspended in an otherwise cation-free medium. Other conditions as for Fig. 4.9.

This observation clearly illustrates that only the net charge is important in determining the type of activity a cation displays in regard to its effect on chloroplast fluorescence yield.

4.2.5. The effect of polyvalent cations on chlorophyll a fluorescence of broken chloroplasts

Divalent cations appear to be more efficient than monovalent cations in creating the high fluorescent state of isolated broken chloroplasts. This effect was shown in the preceding sections to depend solely on the net positive charge carried by the cation. On this basis, it might be expected that polyvalent cations (i.e. of positive valency greater than two) would be even more effective than divalent cations in raising the fluorescence yield of broken chloroplasts from the low yield. To test this idea, several polyvalent cations have been examined for their effects on chloroplast fluorescence. Lanthanum appeared to be a good candidate for such a study since this heavy metal ion appears to be more effective than Ca^{2+} in some biological systems requiring divalent cations for maximal activity, although inhibiting Ca^{2+} effects in others (see Ref. 232).

However, La^{3+} was not observed at any concentration to increase fluorescence of chloroplasts from the low yield created by washing the membranes in EDTA and resuspension in cation-free medium. On the other hand, the trivalent hexaminocobaltic cation did increase fluorescence of chloroplasts under such conditions (Figure 4.13). The maximum yield of fluorescence was seen when approximately 2 μM hexaminocobaltic chloride had been added to the medium. This stimulation of fluorescence was slightly less than half that seen at saturating concentrations of divalent cations. Further addition of hexaminocobaltic chloride induced a rapid decline in chloroplast fluorescence that was not prevented by the presence of 5 mM Mg^{2+} in the medium.

It thus appears that the trivalent hexaminocobaltic cation can increase

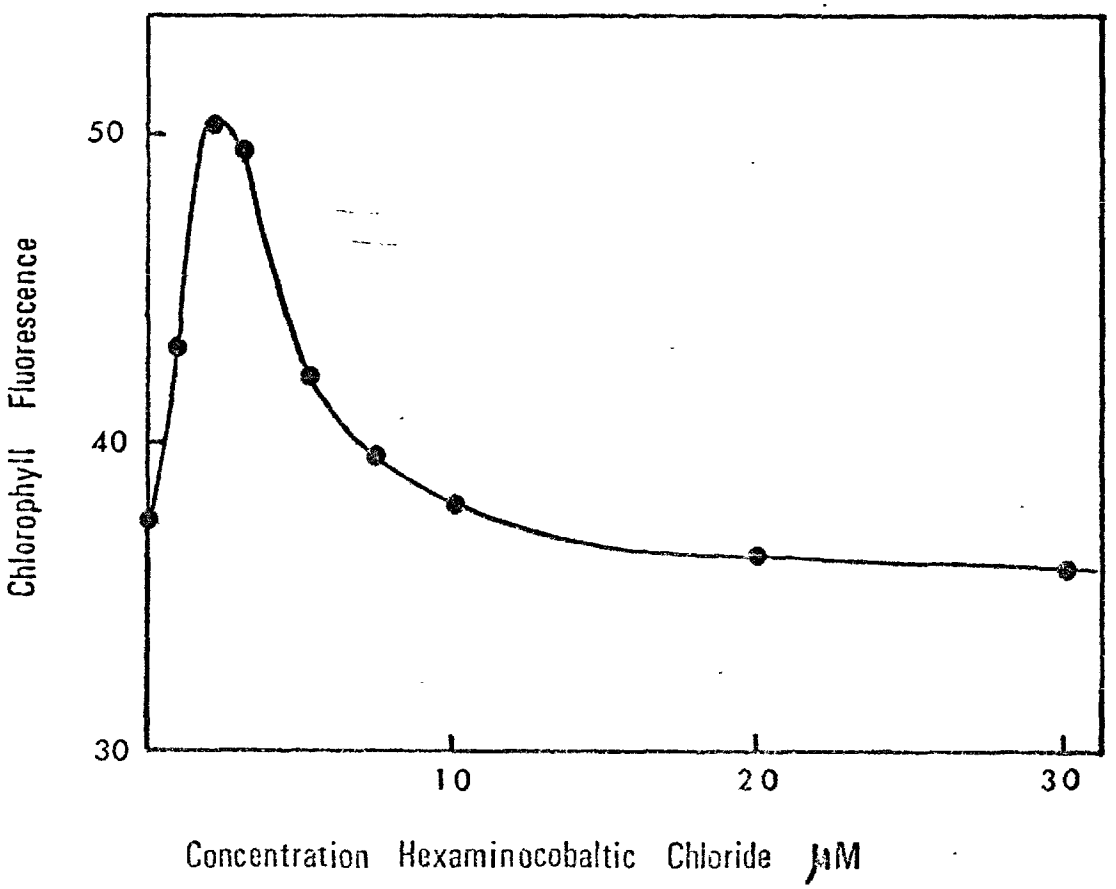


Fig. 4.13 Effect of the trivalent hexaminocobaltic cation on chlorophyll fluorescence from EDTA washed broken chloroplasts suspended in an otherwise cation-free medium. Other conditions as for Fig. 4.9.

chloroplast fluorescence in the manner of a divalent cation, but that this effect is obscured by irreversible quenching of fluorescence also observed for certain monovalent (e.g. NH_4^+) and divalent (e.g. Zn^{2+}) cations. Such irreversible fluorescence quenching at very low concentrations of added cation might account for the lack of effect of La^{3+}. To test this possibility, the effect of La^{3+} on chloroplasts initially in the high fluorescent state was investigated. As shown in Figure 4.14, both La^{3+} and Ce^{3+} at low concentrations ($C_{\frac{1}{2}}$ approximately 10 μM) decreased the high fluorescence of chloroplasts suspended in a medium containing 100 μM Mg^{2+}) as the only added cations. Furthermore, increasing the Mg^{2+} content of the suspending medium to 6.6 mM only slightly increased the $C_{\frac{1}{2}}$ from 10 to 25 μM for both La^{3+} and Ce^{2+} induced fluorescence quenching.

Using the criteria previously introduced for monovalent and divalent cations, such fluorescence lowering by La^{3+} and Ce^{3+} can be termed irreversible. It seems that irreversible fluorescence quenching induced by the trivalent cations so far tested occurs more readily than that induced by certain monovalent and divalent cations. This trivalent cation induced irreversible quenching therefore may partially or totally obscure any additional reversible changes in chloroplast fluorescence.

Irreversible fluorescence quenching may be due to irreversible binding of trivalent cations to the chloroplast. This statement is justified by experiments in which broken chloroplasts were exposed to different cations during the washing stage. Washing the membranes in 10 mM KCl resulted in a low fluorescence yield when the thylakoids were resuspended in a cation-free medium. However, as already shown, fluorescence could be increased from the low level by addition of 5 mM Mg^{2+} or 100 mM K^+ to the medium. However, if 0.5 mM La^{3+} (or Ce^{3+}) were used in place of 10 mM KCl in the washing stage, the resulting fluorescence of chloroplasts suspended in a cation-free medium was fixed at the low yield and could not be increased by Mg^{2+}

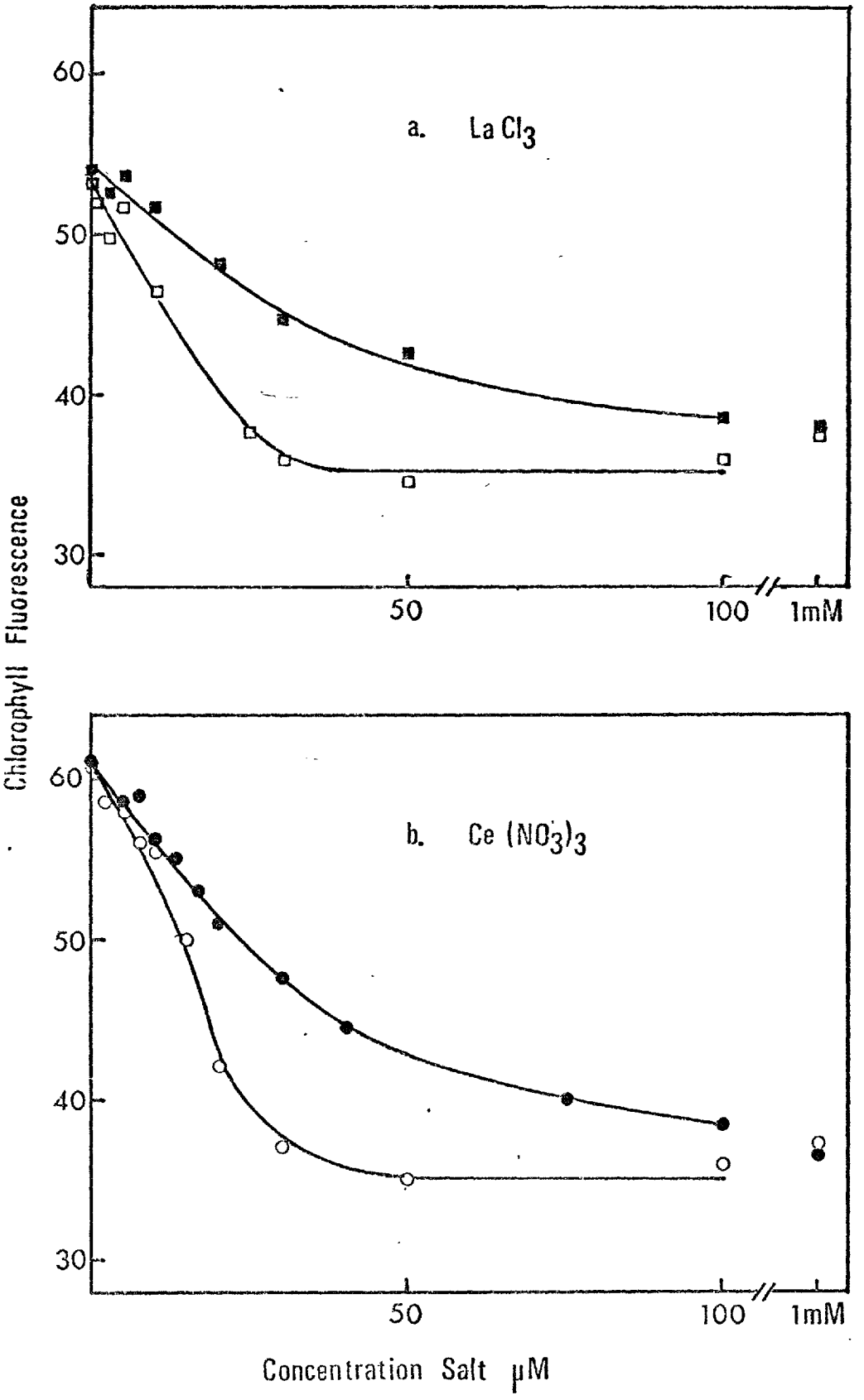


Fig. 4.14 Effect of (a) La^{3+} and (b) Ce^{3+} on chlorophyll fluorescence from broken chloroplasts suspended in cation-free medium containing 13.3µM DCMU and 100 µM $MgCl_2$ (open symbols) or 6.6 mM $MgCl_2$ (solid symbols).Other conditions as for Fig. 4.3.

or 100 mM K^+. This result suggests that La^{3+} or Ce^{3+} binds irreversibly to the thylakoid.

Since lysine has been shown to act as a typical monovalent cation, and lysyl-L-lysine as a typical divalent cation, it was of interest to test the effects of the polymer of this amino-acid for its effects on chlorophyll a fluorescence. Such a study is particularly pertinent in view of the observations of Berg et al.[228] These authors found that poly-L-lysine induced restacking of thylakoids initially suspended in media containing low levels of monovalent cations. Such restacking of thylakoids induced by 5 mM Mg^{2+} or 100 mM K^+ has often been correlated with an increase in chlorophyll fluorescence induced by these cations,[223,224] see Section 4.1.2. The effects of poly-L-lysine on chloroplast fluorescence has not previously been reported.

Table 4.4 shows that poly-L-lysine did not increase the fluorescence of chloroplasts from the low yield created by suspending the membranes in media containing 10 mM monovalent cations. Under these conditions, poly-L-lysine slightly decreased fluorescence and largely prevented the subsequent action of Mg^{2+} (5 mM) or K^+ (100 mM). Thus the reported poly-L-lysine induced restacking of thylakoids suspended in 10 mM monovalent cations[228] is not associated with an increase in fluorescence under these conditions. The action of poly-L-lysine contrasts to that of 5 mM divalent cations and 100 mM monovalent cations which induce both restacking and fluorescence yield increase in broken chloroplasts initially held in low levels of monovalent cations.

Poly-L-lysine is a known inhibitor of electron transport, probably at the point of plastocyanin;[46,235] O_2 consumption of broken chloroplasts in the presence of methyl viologen can therefore be used as additional monitor of the interaction of poly-L-lysine with the thylakoid providing that the electron transport inhibitor DCMU is omitted from the medium. Table 4.4

TABLE 4.4.

The effect of poly-L-lysine on chlorophyll fluorescence and electron transport in broken chloroplasts.

Addition	Fluorescence yield[a] (Arb. units)	Electron transport[b] (Arb. units)
None	100	100
10 mM $MgCl_2$	178	136
180 mM KCl	169	125
0.1 mg/ml^{-1} Poly-L-lysine	90	32
Poly-L-lysine + $MgCl_2$[c]	90	32
Poly-L-lysine + KCl[c]	126	21

(a) Conditions as in Table 4.1.

(b) Chloroplasts suspended in 0.33 M sucrose, 15 mM N-tris(hydroxymethyl)-methyl glycine (tricine/tris pH 7.6 containing 5 mM NH_4Cl, 0.75 mM Na Azide, 15 mM KCl and 60 µM MeVi. For other details see Section 2.5. Rate of electron transport = 100 is equivalent to 110 µatoms O_2 consumed/mg chlorophyll/hr.

(c) Salt added 5 minutes after poly-L-lysine.

shows that light induced electron transport of broken chloroplasts was maximally inhibited (70%) at 0.1 mg/ml poly-L-lysine present in the medium. This inhibition of MeVi dependent O_2 uptake was not relieved when 5 mM Mg^{2+} or 100 mM K^+ was added to poly-L-lysine treated chloroplasts suspended in a medium containing 10 mM K^+. The electron transport data supports the results obtained from fluorescence studies and suggests that poly-L-lysine becomes bound to chloroplasts suspended in low levels of monovalent cations. Such binding does not induce the fluorescence yield changes and blocks the subsequent effects of 5 mM divalent or 100 mM monovalent cations on chlorophyll fluorescence.

When chloroplasts were washed and suspended in a cation-free medium, poly-L-lysine quenched fluorescence from the initial high yield and again blocked the subsequent action of 5 mM Mg^{2+} (Figure 4.15). The fluorescence decrease induced by this impermeable polycation was thus irreversible and analogous to that brought about by La^{3+} and Ce^{3+}. The $C_{\frac{1}{2}}$ for poly-L-lysine induced fluorescence quenching under these conditions was found to be approximately 2 µg ml^{-1} final concentration and to be little affected by the presence of 5 mM Mg^{2+} in the medium. The degree of polymerisation of the poly-L-lysine was approximately 9 residues per molecule, corresponding to an average molecular weight of approximately 2,000. The $C_{\frac{1}{2}}$ for poly-L-lysine induced fluorescence lowering was therefore calculated to be approximately 1 µM, which is 10-20 fold less (on a molar basis) than for La^{3+}.

These results clearly show that the action of lysine on chloroplast fluorescence is extremely dependent on the degree of polymerisation of this amino acid. The monomer (lysine) and dimer (lysyl-L-lysine) act as typical monovalent and divalent cations respectively and bring about reversible fluorescence yield changes in broken chloroplasts. The polymer, however, induces irreversible chloroplast fluorescence quenching at very low concentrations. This would indicate that irreversible quenching of fluorescence,

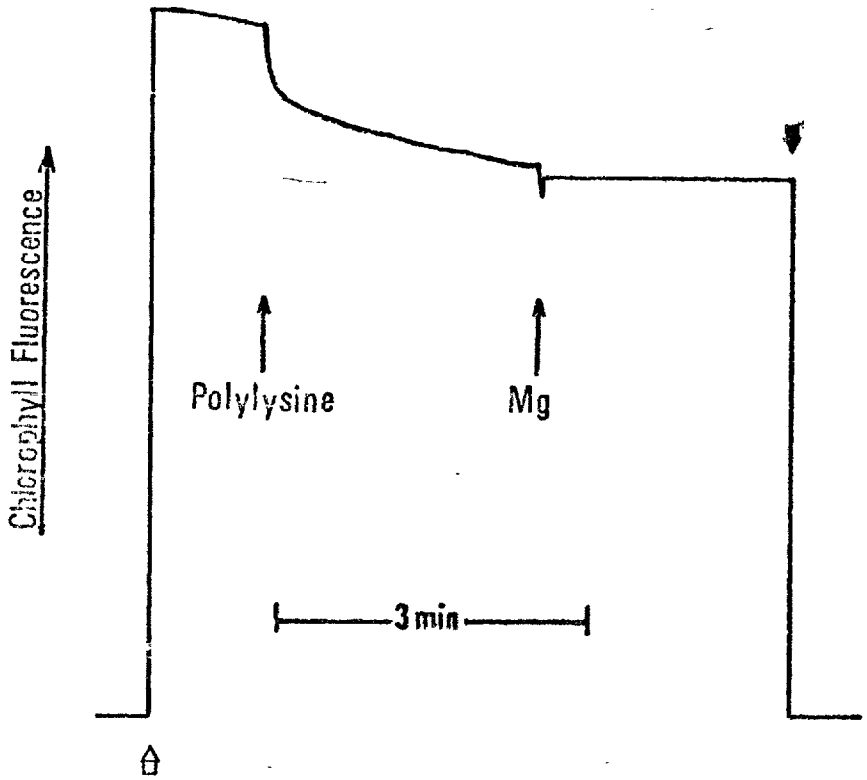


Fig. 4.15 Kinetics of the irreversible chlorophyll fluorescence quenching induced by addition of 0.1 mg ml^{-1} poly-L-lysine to broken chloroplasts washed and resuspended in cation-free medium. Other conditions as for Fig. 4.6.

though a distinct process differing from reversible cation induced fluorescence changes, is nevertheless also very dependent on the net charge born by the cation. Irreversible fluorescence quenching may also therefore be dependent on the initial electrostatic interaction of cations with the thylakoid membrane which may then lead to specific binding of the cation to the membrane (see Section 4.3).

4.2.6. The effect of chloroplast concentration on cation sensitive fluorescence yield changes

The measured $C_{\frac{1}{2}}$ of various cation induced effects on fluorescence can be equated to apparent binding constants describing cation interactions with the thylakoid providing that certain assumptions are made. One assumption is that the interaction between cations and the negatively charged membrane can be described by a simple reversible absorption isotherm. A second is that chlorophyll fluorescence intensity is directly related to the amount of cation bound. A third assumption requires that the amount of cation which becomes bound to the membrane is small in comparison to the amount of cation initially added.

The first assumption presents a mechanism for cation effects on fluorescence which may not be applicable to the observations reported above (see Section 4.3). There is also no *ad hoc* reason for assuming that fluorescence is linear with the amount of cation bound to the thylakoid. For these reasons, I have refrained from equating the $C_{\frac{1}{2}}$'s so far quoted for fluorescence effects to apparent cation binding constants. Evidence is presented in this section that the third assumption stated above may also not be true in certain circumstances.

If a significant proportion of the cation added to the suspension medium of chloroplasts becomes associated with the membranes, then the recorded $C_{\frac{1}{2}}$ from a cation concentration curve is more a measure of the

'titratable sites' controlling fluorescence than a reversible binding constant. Under these conditions, an increase in the amount of chloroplasts in the medium should significantly increase the measured $C_{\frac{1}{2}}$. As shown in Table 4.5, this appears to be the case for La^{3+} induced quenching of chlorophyll a fluorescence when chloroplasts were suspended in a cation-free medium. It can be seen that within the limits of experimental error, a fourfold increase in chloroplast concentration resulted in a fourfold increase in the $C_{\frac{1}{2}}$ for La^{3+} induced fluorescence lowering. This seems to indicate that most of the La^{3+} added to such chloroplasts becomes closely associated with the membranes. It is possible to estimate the total La^{3+} binding capacity of chloroplasts if it is assumed that under these conditions this trivalent cation binds irreversibly to the membrane. That this is so was suggested by washing experiments described in Section 4.2.5. From the data in Table 4.5, such a calculation yields the result that chloroplasts suspended in a cation-free medium bound 6 ± 1 nMole La^{3+} per µg chlorophyll. However, this figure is somewhat variable between different chloroplast preparations. From the data given in Figure 4.14, the amount of La^{3+} bound at half saturation of the fluorescence response (in the absence of 6.6 mM Mg^{2+}) yields a full binding capacity of approximately 2 nMoles La^{3+} per µg chlorophyll for these chloroplasts.

The figure for poly-L-lysine, assuming all of this polycation is bound to chloroplasts in the absence of other cations, is 0.1 nMole per µg, or approximately 1.0 nequivalents per µg chlorophyll. This latter figure was the lowest recorded for any cationic effect on chloroplast fluorescence and corresponds well with the estimate of Gross and Hess.[227] These authors found that the sites controlling fluorescence 'bound' monovalent and divalent cations to the extent of 1.2 nequivalents per µg chlorophyll for chloroplasts suspended in cation-free media.

Since these binding estimates are taken from fluorescence changes

TABLE 4.5.

Effect of chloroplast concentration on $C_{\frac{1}{2}}$ of La^{3+} induced irreversible lowering of fluorescence[(a)].

Chloroplast concentration (μg chlorophyll ml^{-1})	$C_{\frac{1}{2}}$ for La^{3+} induced fluorescence (μM)	
	No other additions	+ 6.6 mM $MgCl_2$
3.3	15 ± 4	240 ± 40
6.7	32 ± 5	330 ± 50
13.3	92 ± 10	310 ± 50

(a) Conditions as for Table 4.2.

induced by cations, it is possible that the higher estimates for La^{3+} include binding of this cation to other sites on the chloroplast not directly involved in the fluorescence yield changes.

In contrast to cation-free conditions, the presence of 6.6 mM Mg^{2+} in the suspending medium caused the relative $C_{\frac{1}{2}}$ for La^{3+} induced fluorescence quenching of broken chloroplasts to be insensitive to a change in chloroplast concentration. Under these conditions, the measured $C_{\frac{1}{2}}$ may be more related to the binding kinetics of La^{3+} to the chloroplast, and not therefore a measure of the amount of La^{3+} bound.

Tables 4.6 and 4.7 show the effect of varying the chloroplast concentration on the $C_{\frac{1}{2}}$ for reversible fluorescence changes brought about by K^{+} and Mg^{2+} respectively. It is evident that a fourfold change in chlorophyll concentration did not significantly affect the $C_{\frac{1}{2}}$ of K^{+} induced fluorescence lowering at low concentrations or the fluorescence increase at high concentrations of this monovalent cation. Such a result would be expected from the high $C_{\frac{1}{2}}$'s for monovalent cation induced effects on fluorescence. If the capacity of the sites controlling fluorescence are of the order 1 nequivalent per µg chlorophyll then only a minute fraction of the K^{+} added (less than 50 µM) would become associated with these sites.

In the presence of 10 mM K^{+} in the medium, the $C_{\frac{1}{2}}$ for the divalent cation induced increase in chlorophyll fluorescence was also quite high (0.4-0.5 mM, Table 4.7). Under these conditions, altering the concentration of chloroplasts in the medium had little significant effect on the measured $C_{\frac{1}{2}}$ for Mg^{2+}. However, for EDTA washed broken chloroplasts suspended in a cation-free medium, the $C_{\frac{1}{2}}$ for the Mg^{2+} induced fluorescence increase was much lower. Under these conditions, it was observed that a fourfold increase in chlorophyll concentration did increase the $C_{\frac{1}{2}}$ for Mg^{2+} (Table 4.7). However, this increase was often found to be less marked than, for instance, the effect of chlorophyll concentration on La^{3+}

TABLE 4.6.

Effect of chloroplast concentration on $C_{\frac{1}{2}}$ for K^+ induced reversible fluorescence yield changes[(a)].

Chloroplast concentration (μg chlorophyll ml^{-1})	$C_{\frac{1}{2}}$ for K^+ induced fluorescence changes (mM)	
	Decrease	Increase
2.8	1.5 ± 0.2	38 ± 5
5.7	1.7 "	36 "
11.3	2.0 "	40 "

(a) Conditions as for Table 4.2.

TABLE 4.7.

Effect of chloroplast concentration on $C_{\frac{1}{2}}$ for the Mg^{2+} induced increase in fluorescence[(a)].

Experiment	Chloroplast concentration (μg chlorophyll ml^{-1})	$C_{\frac{1}{2}}$ for Mg^{2+} induced fluorescence increase (μM)	
		No other additions	+ 10 mM KCl
1	2.2	70 ± 10	480 ± 50
	4.3	180 ± 20	480 "
	8.7	210 "	440 "
2	2.3	40 ± 10	450 ± 50
	4.6	55 "	420 "
	9.3	70 "	490 "

(a) Other conditions as for Table 4.3.

induced fluorescence quenching in the absence of other cations. This result may indicate that under otherwise cation-free conditions, some but not all of the Mg^{2+} added to broken chloroplasts becomes intimately associated with the thylakoid membranes.

The results presented in this section indicate that isolated, broken chloroplasts suspended in media of very low ionic strength can show a high affinity for cations added to the medium. This affinity for cations follows the sequence: poly-L-lysine > La^{3+}, Ce^{3+} > divalent cations >> monovalent cations. Such a sequence is predicted from a consideration of the electrostatic attraction exerted on cations by a fixed negative charge density on the thylakoid membrane as discussed in Section 4.3.

4.2.7. Effect of cations on the binding of the fluorescence probe 9-amino acridine to chloroplasts in the absence of the high energy state

The fluorescent probe 9-amino acridine (9-AA) was shown in Section 3.2.4 to act as a monitor of the light induced energisation of the thylakoid in coupled chloroplasts. However, 9-AA is also a monovalent cation at physiological pH and may be used to probe the interaction of cations with the thylakoid in the absence of the high energy state. It has been reported by Bose[180] that 9-AA binds to chloroplasts suspended in media containing low levels of monovalent cations under dark conditions. This binding of 9-AA was associated with a decrease in the fluorescence of this probe. Addition of $MgCl_2$ (10 mM) under these conditions resulted in release of bound 9-AA from the membrane which could be monitored as an increase in 9-AA fluorescence yield.[180] Since the apparent binding of 9-AA to the thylakoid may well involve the same sites as those controlling chloroplast fluorescence, these effects of cations on 9-AA fluorescence were studied in EDTA washed chloroplasts suspended in cation-free media. High energy state effects on 9-AA fluorescence were eliminated by treating the chloro-

plasts with DCMU and employing low light intensities to excite 9-AA fluorescence (measured at 495 nm, see Section 2.3.1).

As shown in Figure 4.16a addition of Mg^{2+} to chloroplasts suspended in a cation-free medium containing 20 μM 9-AA resulted in an increase in 9-AA fluorescence. The Mg^{2+} induced increase in 9-AA fluorescence corresponds to that reported by Bose[180] although this author used chloroplast suspending media containing low concentrations of K^{+}. If the 9-AA fluorescence increase represents release of bound 9-AA from the thylakoid, then it appears that Mg^{2+} induces this 9-AA release over an identical divalent cation concentration range to that which increases chlorophyll a fluorescence under these conditions.

The effect of monovalent cations on the apparent binding of 9-AA to EDTA washed chloroplasts suspended in an otherwise cation-free medium is shown in Figure 4.16b. It can be seen that 9-AA fluorescence was increased on increasing the level of monovalent cations in the medium with this effect saturating at 100mM added K^{+}. The monovalent cation induced release of bound 9-AA therefore shows significant differences to the effect of these cations on chlorophyll fluorescence in similar conditions. Even in EDTA washed chloroplasts, monovalent cations tend to decrease the chlorophyll fluorescence yield up to a concentration of 10 mM, and at high concentrations restore the yield to the high level. It is pertinent to note that the $C_{\frac{1}{2}}$ of K^{+} induced release of bound 9-AA from EDTA washed chloroplasts was 10 mM. This concentration of monovalent cations is approximately that which creates the minimum chlorophyll fluorescence yield of thylakoids suspended in an otherwise cation-free medium.

If the apparent binding of 9-AA to chloroplasts involves negative sites on the membrane which also control fluorescence, then the results presented above have an important bearing on the way in which metal cations may control fluorescence in these conditions. It seems that divalent

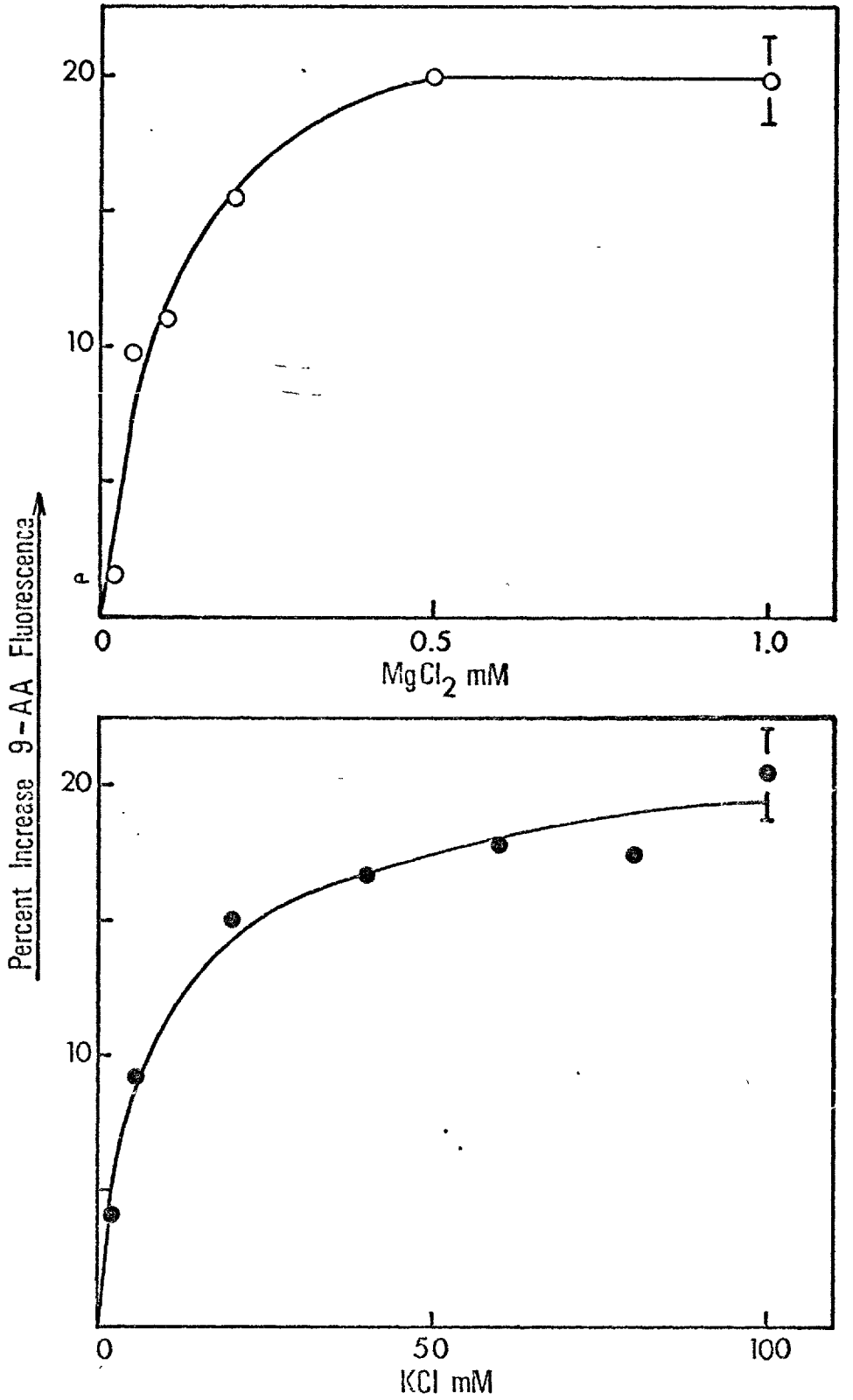


Fig. 4.16 Monovalent and divalent cation induced increase of 9-AA fluorescence observed in the presence of EDTA washed broken chloroplasts (10 μg chlorophyll ml^{-1}) suspended in cation-free medium. All samples contained 10 μM DCMU and 20 μM 9-AA. Fluorescence levels refer to the steady-state relative yields observed after 10 min. incubation in the presence of the appropriate salt, (see Section 2.3.1.)

cations increase chlorophyll a fluorescence under conditions where bound 9-AA is fully released from the thylakoid. Monovalent cations quench chlorophyll a fluorescence under conditions where half the bound 9-AA is released. It is possible therefore that when one divalent cation is associated with each site controlling fluorescence, this emission is high. When one monovalent cation is associated with each site, the fluorescence is low. On increasing the monovalent cation content of the medium to 100 mM, a second monovalent cation becomes associated with the negative site on the thylakoid and chlorophyll fluorescence is restored to the high yield. The association of this second monovalent cation induces release of the remaining 9-AA.

4.3. Discussion

The experiments reported in Section 4.2 have been mainly concerned with the effects of cations on the chlorophyll a fluorescence yield of broken chloroplasts in the absence of a pH gradient across the thylakoids. Those cation induced fluorescence changes which were prevented by the presence of 5 mM Mg^{2+} in the chloroplast suspending medium were termed 'reversible' fluorescence changes.

A striking feature of the studies of reversible fluorescence changes has been the markedly different effects induced by monovalent and divalent cations yet the apparent lack of specificity observed within these two broad groups of ions. It seems that neither the structural nor the lipid solubility properties of cations are important in the mechanism controlling the reversible fluorescence yield changes brought about by these ions. It would appear therefore that cations interact with exposed sites on the outer surface of the thylakoid membrane to effect changes in chloroplast fluorescence. Moreover, the differential effect of monovalent and divalent cations on chloroplast fluorescence were strictly dependent on the net

positive charge born on the cation. This may indicate that fluorescence changes occur through mainly electrostatic interactions between cations and fixed negative charges on the outer surfaces of the thylakoid.

It seems that in the absence of the chloroplast high energy state, the high fluorescing conformation exists when divalent cations are associated with negatively charged sites on the external thylakoid surface. Since overall electroneutrality must be maintained at the membrane surface, broken chloroplasts cannot be freed of these associated divalent cations by simply washing in media containing no other exchangeable cations. Introduction of low levels (10 mM) of monovalent cations into an otherwise cation-free chloroplast suspending medium appears to allow substitution of these associated divalent cations to occur. Under these conditions it seems that displacement of divalent cations at the thylakoid surface by monovalent cations induces a membrane conformational change which brings about the low fluorescent state. Apparently at higher bulk concentrations (100 mM), monovalent cations may interact with the negative sites controlling fluorescence in the same way as divalent cations and the high fluorescence yield is restored. The antagonistic effects of low concentrations of monovalent and divalent cations on fluorescence from chloroplasts suspended in an initially cation-free medium would thus be due to competition between these cations for the negative sites controlling fluorescence.

The latter conclusion was also reached by Gross and Hess.[227] These authors proposed that specific binding of cations to the thylakoid was involved in the mechanism controlling fluorescence. They estimated[227] from Dixon (double reciprocal) plots that the total binding capacity of the sites controlling fluorescence of chloroplasts washed and suspended in a cation-free medium was approximately 0.6 nMoles per µg chlorophyll. Each site apparently bound one divalent cation (high flourescence yield) or two monovalent cations (low fluorescence yield) when low levels (10 mM) of these cations

were added to the chloroplast suspending medium. However, no change in the amount of monovalent cations bound was detectable[227] when the external concentration of these ions was raised from 10 to 100 mM. The model put forward by Gross and Hess[227] cannot therefore explain the intriguing antagonism displayed by low and high concentrations of monovalent cations on chloroplast fluorescence.

In contrast to the view expressed in this thesis, Gross and Hess[227] also concluded from atomic absorption analyses that the sites controlling fluorescence were initially 'empty' when chloroplasts were suspended in a cation-free medium. In their model,[227] the high fluorescence seen under these conditions would not be due to residual divalent cations, but to the lack of any cations associated with the external fluorescence controlling sites. However, this situation would appear to be impossible if such sites contain negative charges. Broken chloroplasts isolated for ionic atomic absorption analysis would be expected to be associated with sufficient positive ions to maintain electroneutrality at the membrane surface.

The conclusion reached by Gross and Hess[227] would imply that cations become specifically bound to the thylakoid through the establishment of coordination bonds with uncharged ligands on the membrane surface. However, as clearly pointed out by Williams,[231,232] such specific binding would be expected to be highly dependent on the ionic size and coordination number of the cation. Depending on their nature and spatial arrangement, cation binding ligands on the thylakoid might be expected to exhibit specificity between K^+ and Na^+ or between Mg^{2+} and Ca^{2+} and certainly to distinguish between organic and metal cations.[231,232] However, except on the basis of net charge on the cation, this specificity was not significantly observed in the experiments reported in this thesis concerning reversible fluorescence yield changes. Mechanisms involving specific cation binding to the thylakoid appear to be inadequate in describing these chloroplast

fluorescence changes.

The results can be better interpreted by considering the interactions of cations with the fixed negative charge density of the thylakoid surface. Such an externally located thylakoid negative charge density would be expected to have a profound influence on the diffusible ions in the surrounding bathing medium. Thus cations would be expected to be attracted to (and anions repelled from) the membrane surface. This effect would result in the formation of an electrical double layer at the membrane/solution interface as shown pictorially in Figure 4.17a. In this model, the negative surface charge layer on the thylakoid is mainly balanced by a diffuse dispersion of positively charged ions in the layer of solution adjacent to the membrane. Such a model was first formulated quantitatively by Gouy and Chapman in the early part of the 20th century and is commonly known as the diffuse double layer theory (see Delahay, Ref. 229). The original Gouy-Chapman theory has since undergone modification and elaboration, but still forms the basis of much modern theory concerned with electrical double layer effects in other branches of science.[229,236-239] Double layer effects have not been previously considered in relation to the various effects of cations on chloroplast structure, chlorophyll a fluorescence yield or other photochemical reactions. However, analysis in terms of simple Gouy-Chapman theory appears to be adequate in accounting for the major observations of cation sensitive chloroplast fluorescence changes reported in this thesis. This theory describes the asymmetric arrangement of diffusible ions in the double layer in terms of two opposing forces acting on charged species in the solution adjacent to the membrane (see Appendix I). At equilibrium, the attractive force exerted by the membrane bound negative charge density (given by the Poisson equation) is balanced by the change in chemical potential (Boltzmann equation) as ions in solution become asymmetrically distributed in relation to their mean bulk concentration.

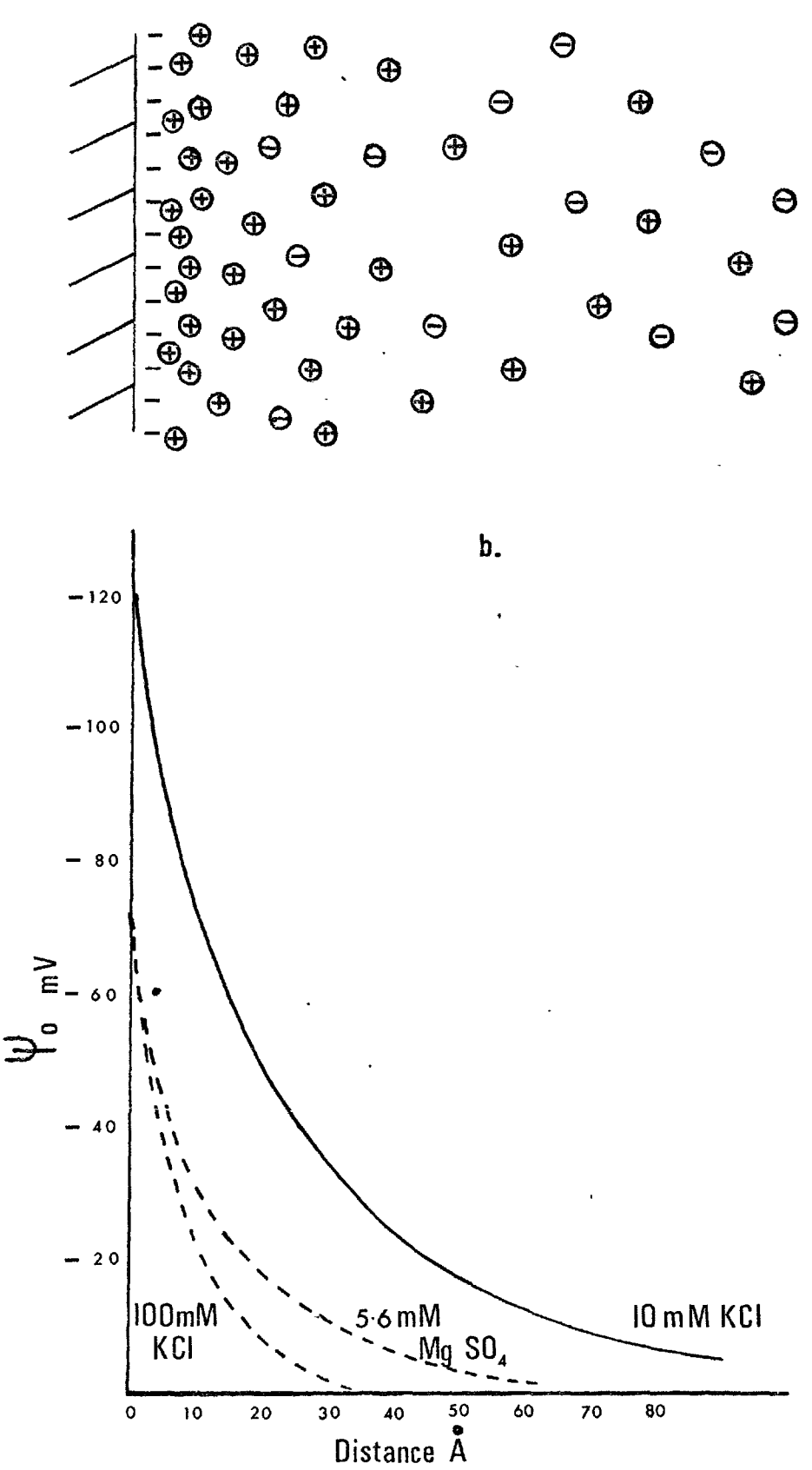


Fig. 4.17 Gouy-Chapman theory of the diffuse double layer: (a) schematic representation of the double layer; (b) surface potential (ψ) relative to the bulk solution and the non-exponential decrease of potential difference with increasing distance (x) from the surface. Theoretical data computed for a negatively charged membrane (6.43 $\mu C\ cm^{-2}$) suspended in a solution containing a single Z-Z electrolyte. See Appendix I and II for details.

At any point within the double layer, therefore, a difference in electrical potential (ψ) exists relative to the bulk solution where ions are assumed to be homogeneously distributed. This potential difference (negative in sign for membranes bearing a net fixed negative charge) has its maximum value at the membrane surface (ψ_o) and decreases towards zero with increasing distance (x) from the membrane surface.

Combination and integration of the Poisson and Boltzmann equations (see Appendix I) leads to an expression linking ψ_o with the average net density of fixed negative charges on the thylakoid (q) suspended in solution containing a Z-Z electrolyte of mean bulk concentration, $c_{i\infty}$:

$$q = \pm \left| \frac{RT\varepsilon}{2\pi} \sum_i c_{i\infty} \left[\exp\left(-Z_i F\psi_o/RT\right) - 1 \right] \right|^{\frac{1}{2}} \qquad \text{Eq. 4.1.}$$

where R,T,F and Z have the usual meaning and ε is the permittivity of water (= 78.5.4.π ε_o at 25°C, where ε_o is the permittivity of free space).

In the presence of a single electrolyte of valency Z-Z in the suspending medium, Equation 4.1 reduces to:

$$q = 2A\,(c_\infty)^{\frac{1}{2}} \sinh\left(ZF\psi_o/2RT\right) \qquad \text{Eq. 4.2.}$$

where $A = \left(\frac{RT\varepsilon}{2\pi}\right)^{\frac{1}{2}}$.

Making the appropriate numerical substitutions for 25°C, equation 4.2 is written:

$$q = 11.74\,(c_\infty)^{\frac{1}{2}} \sinh\left(\frac{Z\psi_o}{51.7}\right) \qquad \text{Eq. 4.3.}$$

where q has units of $\mu C\ cm^{-2}$; c_∞ of Moles l^{-1}; and ψ_o of mV.

It is clear that the magnitude of the negative potential difference existent at the surface of the membrane (ψ_o) is dependent on the average

density of fixed negative charges (q) on the thylakoid. Unfortunately, no reliable quantitative estimate of q exists in the literature. Nobel and Mel[234] attempted to measure q from electrophorescis studies but took pains to use intact chloroplast preparations in their experiments. The assumptions these authors used to derive a value of q of 1 negative charge per 1,600 $\mathring{A}^2$ membrane surface are applicable only to spherical intact organelles.

Their estimate of q refers to the outer chloroplast envelope and cannot therefore be related to the fixed negative charge density on the thylakoid membrane.

It is possible, however, to crudely estimate the magnitude of q by assuming that the cation binding sites which control fluorescence contribute the total fixed negative charge density on the thylakoid surface. Gross and Hess estimated the capacity of these sites as 0.6 nMoles, or 1.2 nequivalents per µg chlorophyll.[227] Barber[75] has previously estimated that broken chloroplasts containing 1 µg chlorophyll have an approximate surface area of 16.7 cm^2 or 16.7 x 10^{16} $\mathring{A}^2$. Combining these two estimates gives an average surface charge density of 1 negative charge per 230 $\mathring{A}^2$ or 6.93 µC cm^{-2}.

On substituting q = 6.93 µC cm^{-2}, it is found that in the presence of 10 mM monovalent electrolyte, ψ_o = -127 mV. Bearing in mind that the estimation of q given above is only very approximately, it seems likely that relatively large negative potentials may exist at the thylakoid surface relative to the bulk suspending medium. As shown in Figure 4.17b, the magnitude of ψ declines rapidly (and non exponentially) with increasing distance x from the membrane surface (see Appendix II). At a distance of less than 100 $\mathring{A}$ from the thylakoid, ψ becomes very small and double layer effects beyond this point can be considered as negligible.

On increasing the level of monovalent cations in the suspending medium to 100 mM, it is found from equation 4.3 that ψ_o is reduced to -71.4 mV.

Thus as the bulk concentration of electrolyte (i.e. at $x > 100$ Å when ψ is very small) is increased, the negative potential difference at the surface (relative to the bulk) is progressively made smaller. Similarly as Figure 4.17b shows the value of ψ at any distance x is also made smaller. Increasing c_∞ also therefore results in a decrease in thickness of the double layer. It is pertinent to note that when divalent cations exist as the only electrolyte in solution, the external concentration required to obtain a value of ψ_o of -71.4 mV is only 5.6 mM. Thus the differential screening effects of monovalent and divalent cations on the negative surface charges of thylakoids may account for the differential concentration requirement of these two groups of ions on chloroplast fluorescence phenomena. Double layer theory also predicts that no specificity between cations within these two groups should be observable. However, the reduction of ψ_o on increasing the levels of monovalent cations in the suspending medium cannot account for the antagonistic effects of low and high concentrations of these cations on chloroplast fluorescence. Since these antagonistic effects were proposed earlier in this section to be due to competition between monovalent and divalent cations for the negative sites controlling fluorescence, Equation 4.1 must be expanded to include mixtures of electrolytes of differing valency.

Before this is done, however, it is of interest to consider what local concentrations of ions can exist in the plane immediately adjacent to a negatively charged membrane surface. Because of the large negative potential (ψ_o) which exists in this plane, double layer theory predicts that the local concentration of cations <u>at the membrane surface</u> would be much higher than those measurable in bulk solution. From the value of ψ_o obtained from Equation 4.3 for a given external concentration of monovalent cations, the local concentration of these cations, c_o, at the membrane surface can be deduced using the Boltzmann equation:

$$c_o = c_\infty \exp(-ZF\psi_o/RT) \qquad \text{Eq. 4.4.}$$

Thus it is found that $c_o \sim 1.4$ M when membranes of surface charge density 6.93 μC cm^{-2} are bathed in 10 mM monovalent cations. On rasing the external concentrations of monovalent cations to 100 mM, ψ_o is reduced from -127 mV to -71.4 mV, and c_o is found to be 1.6 M. Thus it is found that the theoretical local concentration of cations at the membrane surface is nearly constant and independent of the external bulk levels of these ions. This occurs because the increased screening effects on raising the level of monovalent cations in the medium reduces the exponential term in the Boltzmann equation sufficiently to almost completely offset the increase in the concentration term, c_∞. The local concentration of cations at the membrane surface is therefore independent of the bulk levels of these cations in the medium over the concentration range where screening is significant. When q is taken as 6.93 μC cm^{-2}, screening effects are significant when ψ has an appreciable value (> 50 mV), i.e. c_∞ < 100 mM.

It becomes clear therefore that the type of specific binding considered by Gross and Hess,[227] to explain reversible chloroplast fluorescence changes is not applicable to the interaction of cations with thylakoids bearing a significant net charge. Such binding analyses presume that the amount of cation chemically or specifically bound is in equilibrium with cations in bulk solution. However, as indicated above, the local concentrations of cations in solution near the negatively charged membrane surface would be expected to be much higher than those measurable in bulk solution. This would cause serious under-estimation of cation binding constants. However, more important is the calculation that the local cation levels at the membrane surface are constant over the external concentration range which induces fluorescence yield changes in broken chloroplasts. Thus the amount of any cation chemically bound would not be expected to change on

altering the external levels of the electrolyte. McLaughlin *et al*[239] have provided experimental evidence that the amount of Ca^{2+} or Mg^{2+} chemically bound by highly charged, artificial membranes is small and constant over a large external range of divalent cations.

In order to interpret cation induced reversible fluorescence changes in terms of double layer effects, equation 4.1 can be expanded (see Appendix IV) to yield the following expression:

$$\frac{q^2}{A^2} = 4\,c''_{\infty}\left(\cosh^2\left(\frac{F\psi_o}{RT}\right) - 1\right) + 2c'_{\infty}\left(\cosh\left(\frac{F\psi_o}{RT}\right) - 1\right) \qquad \text{Eq. 4.5.}$$

On rearrangement to the quadratic form, this experession allows ψ_o to be determined in mixtures of divalent (c''_{∞}) and monovalent (c'_{∞}) electrolytes:

$$4\,c''_{\infty}\cosh^2\left(\frac{F\psi_o}{RT}\right) + 2\,c'_{\infty}\cosh\left(\frac{F\psi_o}{RT}\right) - \left(4\,c''_{\infty} + 2\,c'_{\infty} + \frac{q^2}{A^2}\right) = 0$$

Eq. 4.6

By holding c''_{∞} constant and solving Equation 4.6 for ψ_o over a range of values for c'_{∞}, it was hoped to reproduce in terms of double layer effects, the fluorescence 'dip' seen on adding monovalent cations to chloroplasts suspended in a cation-free medium. In fact, the term 'cation-free' medium is somewhat misleading since low levels of monovalent cations (approximately 10^{-4}M) in the form of tris cation are initially present. In addition, isolated broken chloroplasts apparently retain low levels of divalent cations in such media. For this reason, c''_{∞} was set at the low levels of 10^{-5} M and Equation 4.6 solved for ψ_o when c'_{∞} was varied over the range 10^{-6} M $< c'_{\infty} < 3.10^{-1}$ M.

Figure 4.18a shows that at and below 10^{-4} M c'_{∞}, ψ_o was maximal and constant and was determined therefore only by the low levels of divalent cations in the external medium. The screening effects of monovalent cations

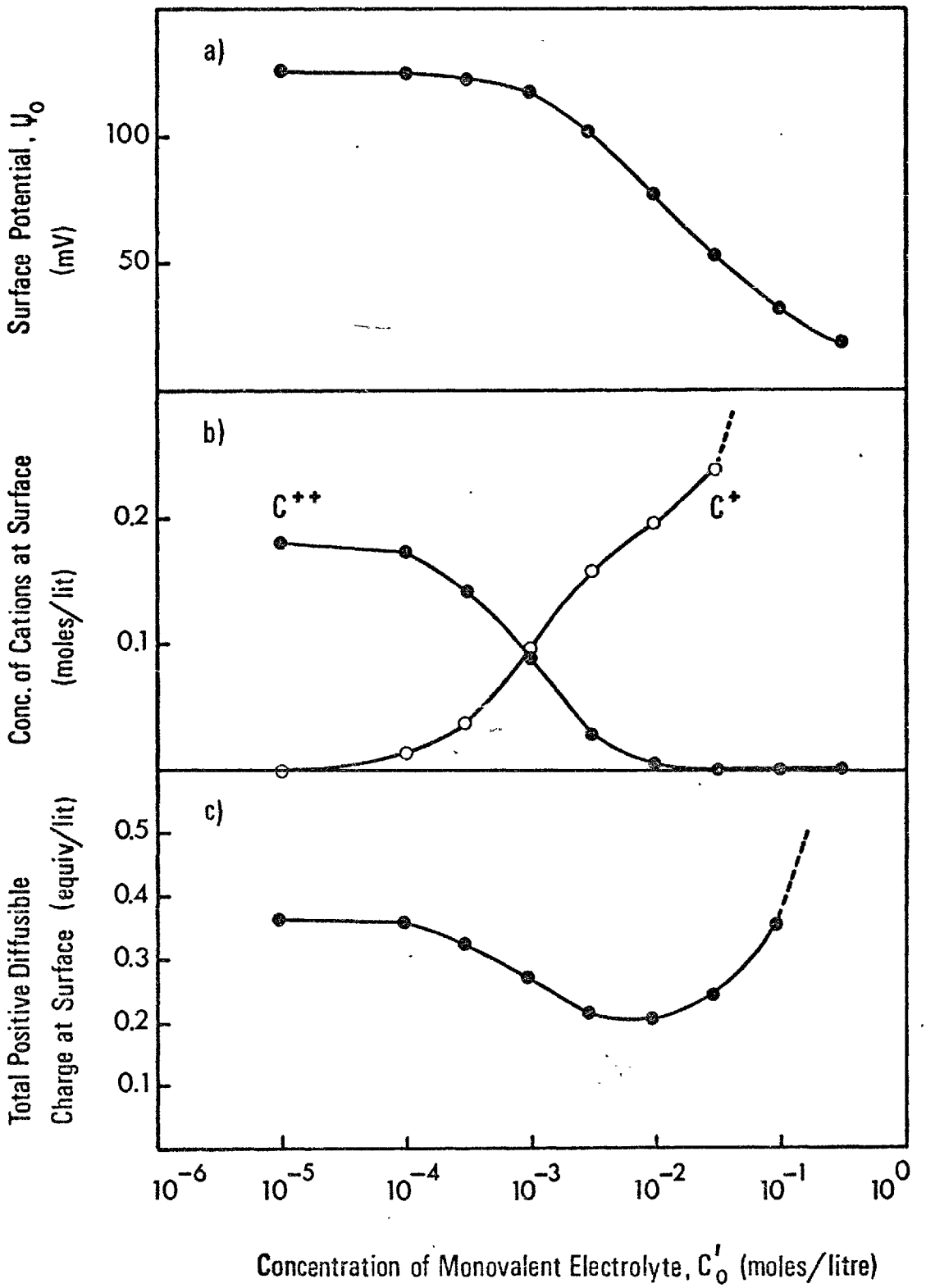


Fig. 4.18 (a) surface potential difference, (b) concentrations of cations in solution adjacent to the surface, and (c) total positive diffusible charge in solution adjacent to the membrane surface as predicted by double layer theory applied to negatively charged surfaces (2.5 $\mu C\ cm^{-2}$) in solutions containing a mixture of monovalent and divalent electrolytes. In this case, divalent cations are held to be constant at a bulk concentration of 10^{-5} M. Note the dip in curve (c) as the theoretical bulk levels of monovalent cations are increased from 10^{-6} to 10^{-1} M. See Appendix IV for details of computations.

(decrease in ψ_o) do not become significant until external levels of c'_∞ are raised above 10^{-3}M. Although again showing the much greater effectiveness on a molar basis of divalent over monovalent cations, these theoretical screening effects do not correlate with the action of these cations on chloroplast fluorescence yield.

It was also found that the calculated local concentrations of cations at the membrane surface, given by the sum of the molar levels of c'_o and c''_o, was independent of external levels of monovalent cations in the range $c'_\infty < 10^{-2}$ M. However, as shown in Figure 4.18b, the relative contribution of divalent and monovalent cations to the total molar levels of those cations at the surface depends on the external levels of monovalent cations. At $c'_\infty = 10^{-4}$ M, mainly divalent cations exist at the surface, and these are replaced by monovalent cations on a one for one basis on increasing c'_∞ to 10^{-2} M. In consequence the total concentration of positive diffusible charge in the plane adjacent to the membrane falls to nearly one half on raising the external levels of monovalent cations from 10^{-4} to 10^{-2} M Figure 4.18c). This effect again results from a decrease in ψ_o which occurs on raising c'_∞ and which is able to offset the increase in the concentration term of the Boltzmann equation. However, on increasing c'_∞ above 10^{-2} M, ψ_o is reduced to a low level. When this occurs, the local concentration of monovalent cations at the surface is mainly determined by the external levels of these cations in the bulk solution. Raising c'_∞ above 10^{-2} M therefore increases both the level of monovalent cations and the total positive diffusible charge concentration at the membrane surface. The latter curve therefore shows a characteristic dip on varying the external concentration of monovalent cations from 10^{-4} to 3.10^{-1} M (Figure 4.18c). The relative position of this dip depends on the value of q taken. Computer analysis indicates that when $q = 2.5 \pm 0.25$ μC cm^{-2}, the theoretical minimum of the positive charge concentration in solution

immediately adjacent to the surface occurs at 5-10 mM c'_{∞}. This would correspond well with observations of the effects of monovalent cations on chloroplast fluorescence.

It seems that the high fluorescing conformation of the thylakoid may be generated when a critical concentration of positive diffusible charge exists in the plane adjacent to the membrane surface. When ψ_o is significant (>50 mV), this critical level is only exceeded when mainly divalent cations exist at the membrane surface. Monovalent cations cannot be sufficiently accumulated, and fluorescence is consequently low, until ψ_o is reduced to a low level and c'_o is determined mainly by external levels of this monovalent cation.

The above hypothesis appears able to account for all the major reversible monovalent/divalent cation effects on chloroplast fluorescence. As shown in Figure 4.20a, on increasing the constant level of divalent cations in the medium from 10^{-5} to 10^{-3} M, the dip in positive diffusible charge at the membrane surface generated on varying the external levels of monovalent cations is progressively abolished. At $c''_{\infty} = 10^{-2}$, no dip is generated and the positive diffusible charge concentration at the membrane surface remains above the critical level for all external levels of c'_{∞} (not shown). This is consistent with the observation that high levels (1-10 mM) of divalent cations in the chloroplast suspending medium prevents fluorescence lowering induced by 10 mM monovalent cations. Under these conditions, fluorescence is maintained at the high yield.

Figure 4.19 shows the set of theoretical curves generated by Equation 4.6 when the external monovalent cation concentration is set constant at 10^{-4} M. On varying the theoretical external levels of divalent cations (c''_{∞}), ψ_o does not become maximal and constant until c''_{∞} is made very low (< 10^{-8} M). Under these conditions ($c''_{\infty} < 10^{-8}$ M), mainly monovalent cations exist at the membrane surface (Figure 4.19b) and the total concen-

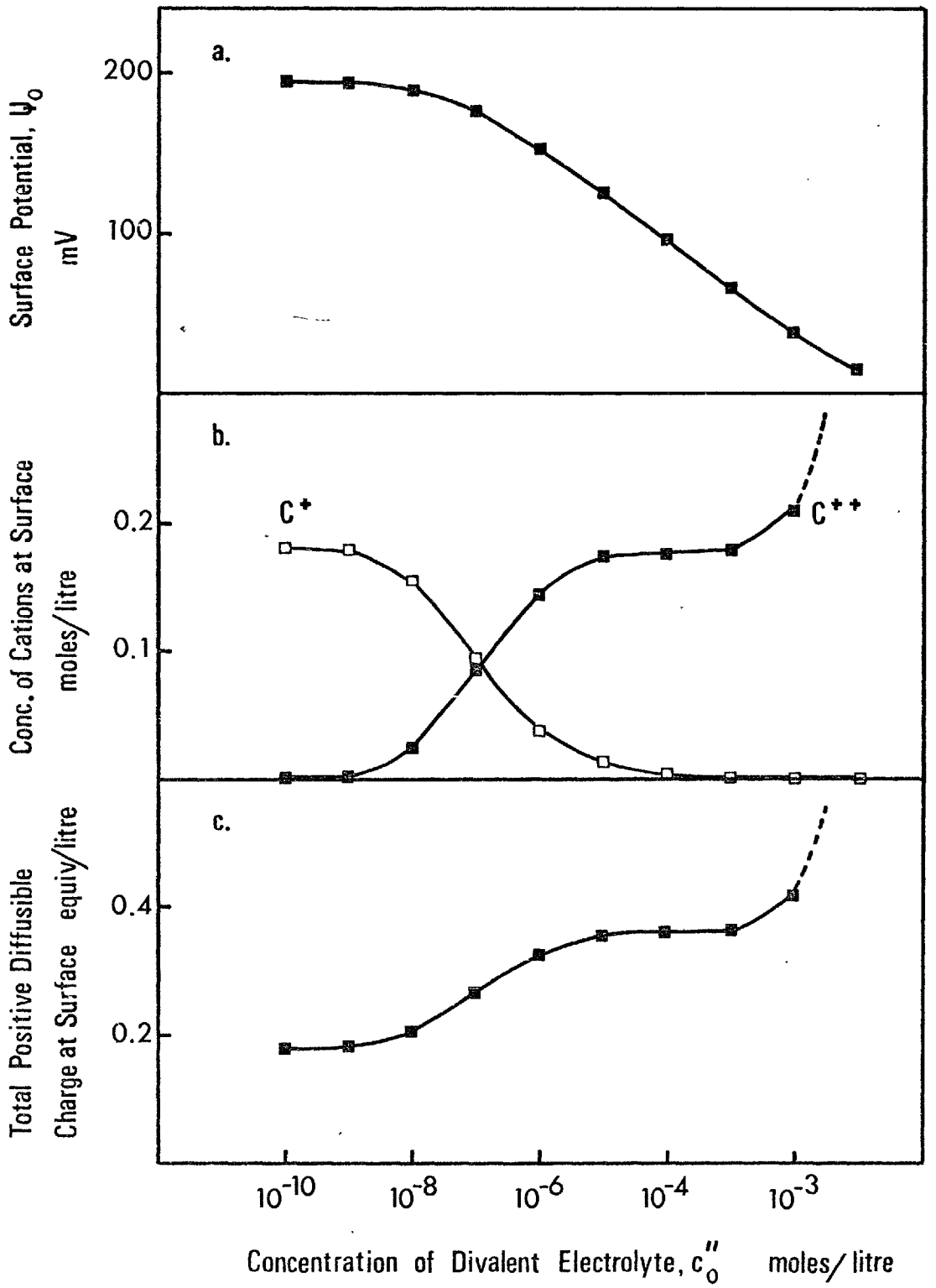


Fig. 4.19 (a) surface potential difference, (b) concentrations of cations in solution adjacent to the surface, and (c) total positive diffusible charge in solution adjacent to the membrane surface as predicted by double layer theory applied to negatively charged surfaces (2.5 $\mu C\ cm^{-2}$) in solutions containing a mixture of monovalent and divalent electrolyte. In this case, monovalent cations are held to be constant at a bulk concentration of 10^{-4} M, and the theoretical bulk levels of divalent cations varied from 10^{-10} to 10^{-3} M.

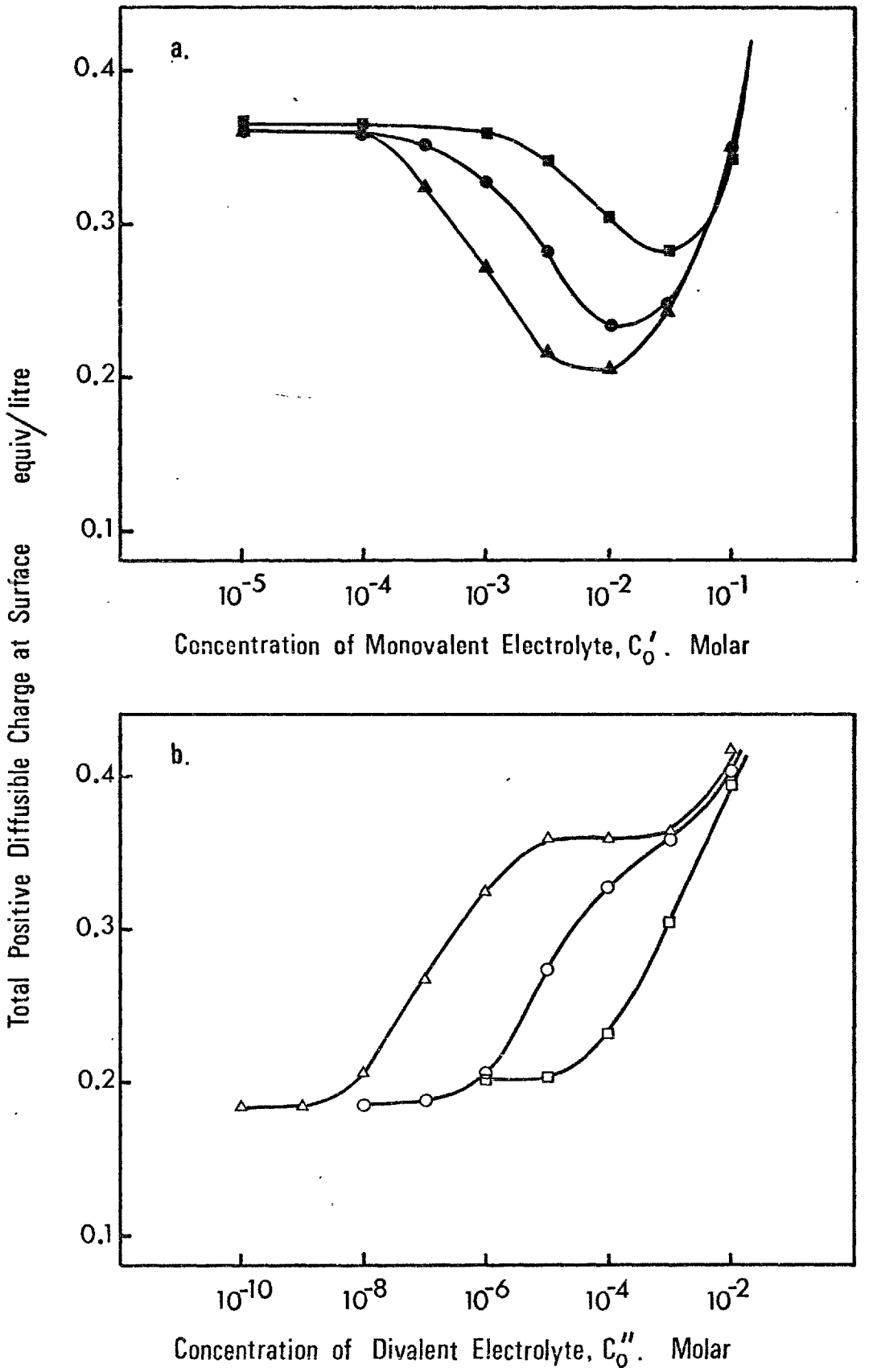


Fig. 4.20 Total positive diffusible charge at the membrane surface as predicted by diffuse double layer theory. (a) Effect of increasing the constant bulk level of divalent cations (triangles, 10^{-5}M; circles, 10^{-4}M; squares, 10^{-3}M) when the bulk concentrations of monovalent cations are varied from 10^{-6} to 10^{-1}M. Note the gradual abolishment of the "dip" of Fig. 4.18 (c). (b) Effect of increasing the constant bulk level of monovalent cations (triangles, 10^{-4}M; circles, 10^{-3}M; squares, 10^{-2}M) when bulk levels of divalent cations are varied from 10^{-10} to 10^{-2}. Note the displacement to higher concentration ranges of the divalent cation induced increase in positive diffusible charge at the surface (i.e. competitive inhibition by monovalent cations). See Appendix IV for details of computation.

tration of positive diffusible charge in this plane is relatively low. This would therefore correspond to a low fluorescing thylakoid conformation. Indeed, the low fluorescing state of broken chloroplasts suspended in 10^{-4} M monovalent cations was only observed on reducing the residual divalent cations in the suspension to a low level. This was achieved by washing the membranes with EDTA. On adding back divalent cations to EDTA washed chloroplasts, the $C_{\frac{1}{2}}$ observed for the fluorescence increase was low (20-50 μM). Increasing the level of monovalent cations in the suspending medium from 10^{-4} to 10^{-2} M competitively inhibited this divalent cation induced fluorescence rise. Figure 4.20b shows that these competitive effects are also predicted by double layer theory. It is clear that an increase in the theoretical external levels of monovalent cations shifts to much high concentrations, the range over which divalent cations replace monovalent cations (and thereby increase the total positive diffusible charge) at the membrane surface. The concentrations at which divalent cations increase the fluorescence of chloroplasts suspended in 10^{-2} M monovalent cations agree well with those predicted by Figure 4.20b. However, at 10^{-4} M monovalent cations the divalent cation induced fluorescence rise was experimentally observed to occur at higher apparent external concentrations than those predicted by double layer theory. Under these conditions, a significant proportion of the divalent cation added to such chloroplasts became closely associated with the membranes. The actual concentration of 'free' divalent cations in bulk solution is therefore probably much lower than that added, and may correspond well to the low levels predicted from Figure 4.20b.

It also seems likely that 9-amino acridine which according to Bose[180] is bound to broken chloroplasts suspended in low salt media, is in fact not chemically bound but rather accumulated in high local concentrations within the double layer. The observed low fluorescence of 9-AA under these

conditions may well reflect concentration quenching effects rather than any chemical binding of the fluorophore to the thylakoid. The differential effect of monovalent and divalent catons in effecting an increase in 9-AA fluorescence would then be due to displacement of 9-AA from the double layer region into bulk solution. The effective dilution of 9-AA which would occur during this process would relieve any concentration quenching effects and increase 9-AA fluorescence yield.

The ability of simple double layer theory to predict the major effects of monovalent and divalent cations on chloroplast fluorescence is somewhat surprising considering the assumptions inherent in the theory (Appendix III). Perhaps the most likely assumption to lead to discrepancies between theoretical predictions and experimental observations is the treatment of diffusible ions as point charges. For high values of q such as are obtained with a mercury electrode,[229] this can lead to impossibly high local concentrations of cations at the membrane surface. However, the relatively low values of q used in the above calculations probably do not render this assumption seriously in error. Typical values of the local concentration of cations at the surface using this low value of q are of the order 200 mM, a quite reasonable figure.

Two further points should be stressed here concerning predictions of Gouy-Chapman double layer theory. Firstly, the only unknown quantities in the generation of theoretical curves such as those shown in Figure 4.18 are the magnitude of q and c''_{∞}. As stated previously, any value taken for the average fixed negative charge density (q) on the membrane leads to generation of theoretical curves of the shape shown in Figure 4.18. The value of q selected (2.5 $\mu C\ cm^{-2}$) gave the best fit to experiment data but was slightly lower than that estimated from the data of Gross and Hess.[227] However, this latter estimation of q involves considerable uncertainty as to the surface area contained in a given amount of chloroplasts. The

discrepancy between the two values of q is not therefore considered sufficient to be of concern.

The value selected for c''_{∞} of 10 μM represents the mean concentration of divalent cations in bulk solution of chloroplasts suspended in a medium containing no added cations. Although this value may be too high under these particular conditions, very similar experimental results were obtained when 100 μM Mg^{2+} was added to the chloroplast suspending medium. Even allowing that most of these added divalent cations became associated with the thylakoids, it seems reasonable to assume that 10 μM 'free' cations may exist in bulk solutions under these conditions.

The second point to be stressed is that Gouy-Chapman theory considers the interactions between ions in solution and the negatively charged membrane surface to be purely electrostatic in nature. In this respect, the theory is similar to Debye-Hückel theory for interaction of hydrated ions in solution. The suggestion that the reversible chloroplast fluorescence changes are due to high local concentrations of monovalent or divalent cations at the membrane surface does not imply subsequent specific binding of cations to the membrane. The fluorescence changes are rather visualised to occur through slow conformational changes in the membrane in response to changes in the local concentration of positive diffusible charge near the surface. Such changes may, for example, be due to local alterations in electrical field strength across the membrane/solution interface.

Due to the need to maintain overall electroneutrality between the thylakoid surface and diffusible cations in the double layer, attempts to isolate 'cation-free' chloroplasts from media of low ionic strength would not be expected to meet with success. Although Gross and Hess concluded that their isolated chloroplast preparations contained sites controlling fluorescence which were initially 'empty', it is unlikely that this is so. Close examination of their data[227] reveals that such chloroplasts in fact

retain relatively significant levels of divalent cations and are characterised by a high initial fluorescence yield.[172] These retained divalent cations would therefore be expected to exist in the plane adjacent to the membrane surface. The remaining attracted cations in the double layer may consist of monovalent tris cations under these conditions. The levels of tris cation retained by such preparations was not, however, estimated by these authors.[227]

Simple double layer theory appears to be successful in quantitatively describing the relatively non-specific (except on the basis of net charge on the ion) effects of monovalent and divalent cations on chloroplast fluorescence. However, the largely irreversible fluorescence quenching induced by trivalent (and relatively high concentrations of certain monovalent and divalent) cations requires some elaboration of this theory. This irreversible quenching of fluorescence was not prevented by the presence of 5 mM Mg^{2+} in the chloroplast suspending medium and may involve specific binding of cations to the thylakoid membrane.

The mechanism by which such specific binding might occur is, at this stage, not entirely clear. The extent of any specific adsorption of cations onto the thylakoid should, however, be dependent on the local concentrations of these species at the thylakoid surface. It would be expected therefore that the initial attraction of the membrane surface for the cations in solution would be important in the irreversible fluorescence quenching process. It was in fact observed that the relative effectiveness of cations which induced irreversible fluorescence quenching was highly dependent on the net positive charge on the ion. This is clearly shown by comparing the concentrations of poly-L-lysine (2 μM) required to induce irreversible fluorescence quenching with that for lysine (> 50 mM). The irreversible fluorescence quenching phenomena may also therefore depend on high local concentrations of these cations which occur at the thylakoid in response to

the negative potential at its surface. Unlike reversible fluorescence changes, irreversible fluorescence quenching may occur by subsequent specific adsorption of cations onto the thylakoid surface. Specific adsorption may occur through the formation of coordination complexes between cations and the fixed negative charges on the thylakoid. However, this seems unlikely since there was no clear correlation between the ionic size and coordination number of the cation and its ability to induce irreversible fluorescence lowering.

However, as clearly stated by Ansen,[240] an important consideration in specific adsorption onto the charged mercury electrode appears to be the magnitude of the hydration energy of the absorbed ions. Small and relatively highly hydrated alkali and alkaline earth cations exhibit little or no specific adsorption because their relatively high hydration energies oppose this process.[240] This idea can be extended to observations made with thylakoid membranes. It appears that reversible fluorescence yield changes clearly observed with alkali and alkaline earth cations may be due to electrostatic interactions between hydrated cations and hydrated negative charges on the membrane as discussed earlier. On the other hand irreversible fluorescence quenching may be due to formation of dehydrated ion pairs (or possibly coordination complexes) between cations and the negative charges on the thylakoid. This latter process would be favoured by weaker hydration energies on the cation (e.g. organic cations) or strong electrostatic attraction between the negatively charged membrane and polyvalent cations.

The effect of specific binding can be thought of as an effective reduction of the value of q, the negative charge density on the thylakoid. This can be most clearly visualised by considering protonation of negative sites on the thylakoids as specific binding of H^+. As shown in Section 3.2.4, lowering the external pH of the chloroplast suspending medium brings about

Mg^{2+} insensitive fluorescence lowering. Irreversible fluorescence quenching brought about by an effective reduction in the magnitude of q would also involve a decrease in field strength across the membrane solution interface. Whether or not this lowered field strength is the ultimate cause of fluorescence lowering, as proposed for reversible effects of 10 mM monovalent cations on fluorescence, must be the subject of further investigation. However, it seems reasonable at this stage to tentatively propose that the same external fixed negative charges on the thylakoid are involved in both reversible and irreversible cation induced fluorescence changes.

It also appears likely that the thylakoid negative charge density is involved in the membrane stacking phenomena. As discussed in Section 4.1.2, stacking of isolated broken chloroplasts shows an identical dependence on the ionic constitution of the suspending medium as does the reversible high and low chlorophyll flourescence state. On addition of low levels of monovalent cations to chloroplasts suspended in a cation-free medium, thylakoid unstacking is observed to occur.[224] According to double layer theory, this monovalent cation induced unstacking would correspond to a decrease in positive diffusible change in solution at the membrane surface. Such a decrease in the local concentration of positive charge at the membrane surface might be expected to increase the thickness of the double layer (i.e. increase ψ at distances further away from the membrane surface). An increase in double layer thickness would in turn tend to increase the repulsive forces between membranes and thereby promote unstacking. However, further addition of monovalent (100 mM) or divalent (5 μM) cations would again reduce the thickness of the double layer and bring about restacking of the membranes. Qualitatively, therefore, it seems that cation induced structural changes in broken chloroplasts such as those involving membrane stacking may be due to changes in the structure of the electrical double layer. However, quantitative analysis of cation induced changes in double

layer thickness must for the moment be deferred because of the difficulty in integrating the appropriate equations applicable to mixed electrolytes.

This qualitative picture can be extended to include the action of cations such as poly-L-lysine, which mainly induce irreversible quenching of chloroplast fluorescence. Poly-L-lysine, like 5 mM divalent or 100 mM monovalent cations, induces restacking of thylakoids initially suspended in media containing low levels of monovalent cations.[228] It seems therefore that specific binding of poly-L-lysine to the thylakoid also reduces the thickness of the double layer. This would be consistent with the proposal made earlier that such specific binding effectively reduces the magnitude of q, the fixed negative charge density on the membrane.

It was observed, however, that poly-L-lysine induced restacking of thylakoids does not result in the high fluorescence state seen in the presence of 5 mM divalent or 100 mM monovalent cations. Thus both thylakoid stacking and chlorophyll fluorescence changes seem to represent aspects of cation induced changes in the structure of the double layer. However, it is apparent that the mechanism involved in the fluorescence changes is not identical to that involved in stacking. Berg et al[228] have recently provided evidence that the fixed negative charges on the thylakoid involved in stacking phenomena (and hence in fluorescence changes) consist mainly of carboxyl groups. These authors[228] modified the thylakoid membrane carboxyl groups by carbodiimide activation followed by glycine methyl ester substitution, a procedure which leaves the derivatised groups uncharged. This charge alteration resulted in chloroplast preparations which remained stacked even in the presence of low levels of monovalent cations. Furthermore, the capacity of the derivatised membranes to 'bind' poly-L-lysine was much reduced.[228] These observations provide further support for the above proposal that thylakoid stacking changes occur through changes in the thickness of the double layer associated with membrane surface charges.

It appears that any treatment which reduces the double layer thickness below a critical level causes the thylakoids to be stacked. These treatments include an effective reduction in the magnitude of q by specific binding of poly-L-lysine, chemical esterification of carboxyl groups, or high screening of thylakoid negative charges in the presence of 5 mM divalent of 100 mM monovalent cations in the suspending medium. On the other hand, thylakoid fluorescence changes seem to be closely related to the electrostatic interactions resulting from the high levels of positive diffusible charge at the membrane surface and do not always correlate with changes in the thickness of the double layer.

It has long been known that much of the lipid complement of thylakoid membranes consists of uncharged mono-and digalactosyl diglycerides.[241] It would seem likely, therefore, that most of the carboxyl groups which apparently constitute much of the fixed negative charge density on the thylakoid are contributed by membrane bound proteins. Furthermore, it has been estimated[58] that as much as 50% of the protein in the thylakoid may be present in the chlorophyll a/b light harvesting pigment protein complex. It is tempting to speculate therefore that cation induced fluorescence changes are brought about by structural rearrangements of the chlorophyll a/b light harvesting complex in the thylakoid membrane. These structural rearrangements might be directly due to interaction of cations (via the double layer) with negative charges on the light harvesting complex which are exposed on the outer surface of the thylakoid membrane.

Such a model may have important implications in the regulation of the distribution of light energy between PSI and PSII both <u>in vivo</u> and in isolated, broken chloroplasts. According to Butler and Kitajima,[128] cation induced changes in spillover (see Section 1.2.6) observed in broken chloroplasts may be due to changes in the transfer of absorbed excitation energy from the chlorophyll a/b light harvesting pigment to PSII and PSI.

A low chloroplast fluorescence yield would be due to significant transfer of excitation energy absorbed by the light harvesting pigment to the weakly fluorescence chlorophyll of PSI. Excitation energy absorbed by PSII may also be transferred to PSI (spillover) via the light harvesting pigment complex under these conditions. On the other hand, the high chloroplast fluorescing state would result from transfer of excitation energy absorbed by the light harvesting complex mainly to the highly fluorescence chlorophyll of PSII.[128] The fluorescence increase seen on adding 5 mM divalent or 100 mM monovalent cations to chloroplasts suspended in low levels of monovalent cations would therefore represent an increase in distribution of absorbed excitation energy to PSII at the expense of PSI. Such a cation induced inhibition of spillover may thus be caused by changes in the position or orientation of the chlorophyll a/b protein complex in the membrane. These changes in turn would be proposed to occur via cation induced changes in the local electrostatic forces acting across the thylakoid /solution interface described by double layer theory.

The above discussion has been concerned with the effects of cations on broken chloroplasts in the absence of the light induced high energy state. Under these conditions, cations appear to interact with fixed negative groups on the outer side of the thylakoid membrane via the electrical double layer.

In Section 3.3 however, it was concluded that establishment of a light induced pH gradient across the thylakoid appeared to promote apparent spillover of excitation energy from PSII to PSI and consequently bring about a lowering of fluorescence. These changes were proposed to occur through protonation of fixed negative charges on the inner side of the thylakoid membrane. These results can now be integrated with those discussed in this section by a schematic model of the thylakoid membrane such as shown in Figure 4.21. In this model, the chlorophyll a/b light harvesting pigment

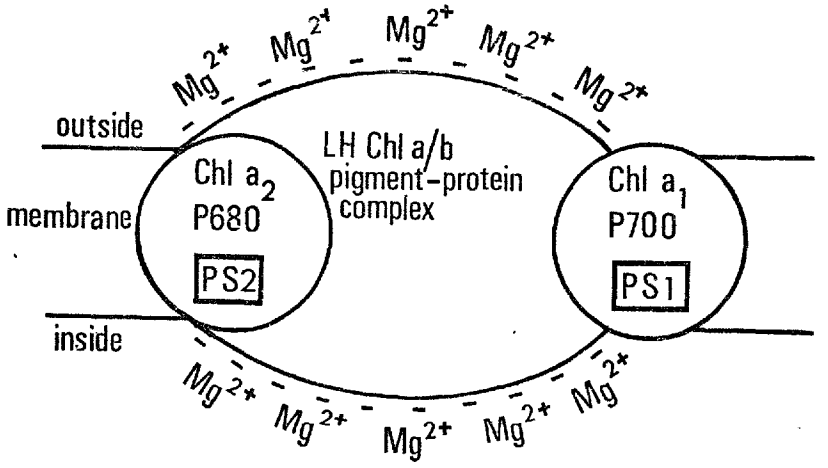


Fig. 4.21 Schematic diagram of the arrangement of chlorophyll a in the thylakoid membrane. In vivo chlorophyll a is proposed to be functionally divisible to three groups (i) that associated with the PSII (PS2) reaction centre; (ii) that associated with the PSI (PS1) reaction centre; (iii) that associated with the light-harvesting chlorophyll a/b pigment protein complex. The light harvesting pigment protein complex is proposed to expose fixed negative charges on either side of the thylakoid membrane. The interaction of monovalent and divalent cations with externally located charges, or light induced protonation of the internally located charges may induce conformational changes in the light-harvesting pigment protein complex which in turn may alter the relative distribution of incoming light energy to the reaction centres of PSII and PSI.

protein complex is proposed to span the membrane (as discussed by Anderson, see Ref. 57) and expose negatively charged regions at both the inner and outer thylakoid surfaces. In dark adapted membranes, the high fluorescence state would be created when a high diffusible positive charge exists in solution adjacent to the external thylakoid surface. This occurs when broken chloroplasts are suspended in low levels of divalent or high levels of monovalent cations. In the intact chloroplast, the stromal levels of Mg^{2+} are apparently high enough to satisfy this condition.

However, a negative charge density on the inner side of the thylakoid membrane would also be expected to be associated with a diffuse layer of attracted cations. It is not clear at this stage whether low levels of divalent or high concentrations of monovalent cations are required on both sides of the thylakoid to bring about the high fluorescing state. However, observations that large, impermeable cations such as lysine, lysyl-L-lysine and choline increase fluorescence of broken chloroplasts suspended in low salt media would suggest that only the external sites are involved in these cation induced fluorescence changes.

Light induced fluorescence quenching due to the establishment of the high energy state would involve protonation of the negative groups on the inner side of the membrane. This in turn induces conformation changes in the membrane and/or chlorophyll a/b light harvesting complex which promote transfer of excitation energy to PSI at the expense of PSII. This would account for State I to State II transition *in vivo*.[155]

Light induced high energy state dependent fluorescence quenching in intact chloroplasts isolated from peas and spinach does not seem to involve changes in the association of cations with the fixed negative charges on the outer side of the thylakoid membrane.[192,242] Telfer *et al* have found that thylakoids within isolated intact chloroplasts remain stacked throughout a period of light induced fluorescence quenching and subsequent reversal

to the high yield observed on darkening the sample.[192] Vernotte et al have also presented evidence that light induced quenching of fluorescence from chloroplasts in the intact leaf is not associated with changes in thylakoid stacking.[242] It would seem, therefore, that at all times the stromal level of cations (probably Mg^{2+}) are sufficient to maintain relatively high concentrations of positive diffusible charge adjacent to the externally located thylakoid surface. Any increase in stromal levels of Mg^{2+} brought about by efflux of this cation from the intrathylakoid space in response to light induced proton pumping does not seem to be important in the associated fluorescence changes of intact pea chloroplasts. However, such a light induced increase in stromal Mg^{2+} may be involved in the activation of certain enzymes concerned with CO_2 fixation (see Section 1.1.7).

In vivo, the State II to State I transition can be brought about by superimposing PSI light onto algae preilluminated in PSII light to initially generate the State II condition.[155] The State II to State I transition is characterised by an increase in chlorophyll fluorescence yield and an inhibition of spillover. It would thus appear that this transition involves a decrease in the magnitude of the transthylakoid high energy state in vivo. It is not immediately apparent, however, how this might occur. The dependence of the State II to State I change on PSI light implicates a role for PSI dependent cyclic electron transport in the process. However, initiation of proton pumping linked to cyclic electron transport on illuminating algae with PSI light would be expected to increase the magnitude of the thylakoid high energy state and thereby increase fluorescence quenching.

In order to effect a decrease in the extent of the ΔpH gradient across the thylakoid, it may be proposed that cyclic electron transport normally operates even in PSII light but becomes inhibited on superimposing PSI light onto in vivo chloroplasts. Although at first sight this statement appears illogical, there is some justification for such a proposal.

Telfer et al[243] have found that PSI dependent cyclic electron transport can contribute to the high energy state induced fluorescence lowering observed on illuminating intact chloroplasts with broad-band green (PSII) light. This light-induced fluorescence quenching supported by cyclic electron flow was, however, only occasionally observed and was inhibited by DCMU at higher concentrations than those required to block non-cyclic electron transport.[243] The inhibitory action of DCMU appears to reflect the fact that efficient cycling of electrons around PSI requires a subtle poising of the redox state of electron carriers intermediate between PSII and PSI.[49] Overall oxidation of these intermediate electron carriers, such as occurs in the presence of DCMU at high concentrations, inhibits PSI dependent cyclic electron flow.[49] These results may become significant when considering that the State II to State I transition in vivo requires relatively high intensities of PSI light.[187] These high intensities of PSI light would also be expected to cause overall oxidation of intermediate electron transport carriers between PSII and PSI, and this could conceivably inhibit any cyclic electron transport. The fact that State II to State I changes were relatively small, only occasionally observed in isolated intact chloroplasts and not observed at all in broken chloroplast preparations (see Section 3.2.7) would indicate that the mechanism 'switching' electron flow from cyclic to non-cyclic modes is extremely labile. It must be noted, however, that there is little experimental evidence for such a hypothetical scheme.

An alternative mechanism to explain State II to State I transitions observed in vivo has been proposed by Barber in Ref. 199. This mechanism involves activation of cyclic electron flow coupled to proton pumping which is proposed to occur on superimposing PSI light onto algae pre-illuminated with PSII light. It was visualised that increased proton pumping under these conditions would lead to increased rates of phosphorylation of ADP to ATP which in turn might increase the ATP/NADPH ratio in the stroma. In turn, the increased ATP/NADPH

ratio may trigger a hypothetical ATP'ase which would wastefully dissipate the high energy state of the chloroplast through the creation of an overall futile cycle. Again, however, no experimental evidence exists to support such a scheme as the cause of State II to State I transition.

A third possible explanation for the State I to State II transition might involve changes in the interaction of cations at the externally located thylakoid sites controlling fluorescence and spillover. Although such changes do not seem to occur in intact pea and spinach chloroplasts, Bennoun and Jupin[244] found that such changes may occur in the green algae Chlamydomonas. These authors found that when these algae were preilluminated to create the State I condition, the in vivo thylakoids appeared to be more extensively stacked than in algae preilluminated to State II. It seems therefore that the State II to State I transition might occur through an increase in the stromal levels of Mg^{2+} or other divalent cations induced by illumination of the algae with PSI light. If this is so, then in vivo chloroplasts of Chlamydomonas, unlike pea or spinach, would not appear to maintain sufficient stromal levels of these cations in the dark to create the initial high fluorescing state. However, the stromal levels of Mg^{2+} within the chloroplasts of Chlamydomonas may be increased in the light by efflux of this cation from the intrathylakoid space in response to proton pumping. Again, therefore, the adaption of Chlamydomonas to State I or State II would depend on the magnitude of the light induced high energy state. This dependence would be very subtle indeed, since any light induced increase in fluorescence brought about by increased stromal levels of Mg^{2+} acting at the external thylakoid sites would be opposed by high energy state quenching of fluorescence via the internal sites on the thylakoid membrane. The high initial stromal levels of Mg^{2+} in isolated pea and spinach chloroplasts would therefore explain why State I to State II changes are not observed in these latter preparations.

Although several alternative mechanisms have been presented above, it is clear that none of these provides a totally satisfactory explanation for the State II to State I adaptive response observed in *in vivo* chloroplasts illuminated in PSI light. Further studies are required to establish the relative merits of these alternative models. Further investigation should include comparative studies to establish that State I/II responses observed mainly with unicellular photosynthetic algae[155,156,177,187] are common to higher forms of plant life such as spinach. It is then necessary to carefully analyse the ionic content of isolated intact spinach chloroplasts which show the full state I/II changes under appropriate illumination conditions.

These studies should be combined with estimates (of the highest possible precision) of the relative magnitude of the high energy state maintained by isolated, intact chloroplasts under illumination which brings about State I or State II. It is to be hoped that these measurements may eventually be made using isolated intact chloroplasts from unicellular organisms such as *Chlorella* and *Chlamydomonas*. This would eliminate the present unfortunate necessity of having to correlate State I/II changes studied in one species *in vivo* to possible light induced ion fluxes within intact chloroplasts isolated from an unrelated species.

A novel feature of the work presented in this thesis has been the application of simple double layer theory to successfully account for the various effects of cations on chlorophyll *a* fluorescence yield in isolated broken chloroplasts. From these studies, it was estimated that the outer surface of the thylakoid membrane bears an average net negative charge density of 2.5 ± 0.25 $\mu C\ cm^{-2}$. Several effects of cations on artificial lipid membranes, which can bear much higher fixed negative charge densities on their surfaces, have also been successfully explained in terms of double layer theory. For example, ionophore facilitated transport of cations

across negatively charged lipid bilayers[239] seems to be affected by the surface potential (ψ_o) which is determined by bulk concentrations of divalent cations in the suspending medium. Changes in surface potential (screening effects) have also been implicated in structural changes in artificial membranes brought about by altering the levels of cations in the suspending medium. These cation induced structural changes include reorganisation of the lipids ('phospholipid flip-flop'[245]) and isothermal phase transitions[246] in the membrane.

The existence of a net negative charge density on the thylakoid and the resulting negative surface potential difference relative to the bulk solution must also be considered in other areas of photosynthesis study. For example, a significant negative surface potential would be expected to reduce the concentration of diffusible anions in solution adjacent to the membrane surface to very low levels. This may be of importance in the study of phosphorylation, since ATP, ADP and Pi all bear an overall negative charge. The fact that thylakoids bind these cofactors of phosphorylation with an apparently high affinity[65] would suggest that double layer effects are not important in this instance. It could be that the sites concerned with ATP synthesis (coupling factor) do not bear an overall net negative charge, or are so positioned on the membrane so as not to be significantly affected by the negative potentials in the double layer.

The expected repulsion of anions from the thylakoid surface may also be an important consideration in electron transport studies which utilise negatively charged electron acceptors such as ferricyanide and $NADP^+$. Walz *et al*[247] have found that the rate of light induced ferricyanide reduction by broken chloroplasts suspended in a glycine medium was dependent on the cation content of the medium in a manner analagous to the chlorophyll fluorescence changes reported in Section 4 of this thesis. It seems that a high rate of ferricyanide reduction occurred under

conditions where the concentrations of positive diffusible charge in solution adjacent to the membrane surface might be expected to be relatively low. It is possible that the rates of electron transport measured by Walz et al[247] reflect cation induced changes in spillover rather than direct effects of ψ_o on the reduction of ferricyanide by light induced electron transport reactions. However, cation induced changes in the rates of PSII and PSI partial electron transport reactions have recently been reported by Gross et al[248] which did not correspond to changes in fluorescence observed under similar conditions. These latter authors, however, did not include glycine in their chloroplast suspending media.[248]

As discussed in Section 1.3, such disagreements between different authors as to the effects of cations on the rate of light induced electron transport in broken chloroplasts are common in the literature. It is clear that comprehensive studies are required in this area of research in order to ascertain the reasons for the varying and often conflicting results. It should be noted that the rate of electron transport measured under a particular set of conditions will be influenced by a number of variable parameters including the magnitude of the chloroplast high energy state, relative light intensity, and possibly the magnitude of ψ_o as well as the degree of spillover of excitation energy between PSII and PSI. It is possible that varying the level of cations in the chloroplast suspending media might influence the rate of electron transport both through changes in spillover and the absolute magnitude of ψ_o. Only when such considerations are taken into account to design carefully controlled experiments can studies of electron transport reactions be unequivocably used to evaluate the validity of the spillover hypothesis.

The existence of fixed negative charges on both the inner and outer surfaces of the thylakoid membrane may also lead to static potential gradients across the thylakoid if the charge densities of the two surfaces

differ. Such a static potential gradient would be present in dark adapted thylakoids and would be sensitive to changes in the bulk concentrations of cations in the suspending medium. Optical density changes at 515 nm[31] or delayed light emission,[72] both of which have been used to study transthylakoid electrical potentials generated in the light, might also be used to investigate the existence of static potential gradients across dark adapted thylakoids.

Finally, the relatively small intrathylakoid volume of chloroplasts might cause double layers associated with the inner thylakoid surfaces to overlap in space. The degree of overlap would depend on the thickness of the internally located double layer, and again would be expected to be decreased on increasing the bulk concentration of cations in the external thylakoid suspending medium. The existence of overlapping double layers within the thylakoid may be responsible for two other observable properties of isolated broken chloroplasts. Firstly, the opposing internal walls of a thylakoid would tend to repel each other and this could partially determine the volume of the intrathylakoid space. Increasing the levels of cations in the suspending medium would reduce any electrostatic repulsion, with divalent cations again being much more effective than monovalent cations. This may give rise to apparent shrinkage of thylakoids by 'non-osmotic mechanisms' as observed by Packer and colleagues[223,225] (see Section 4.1.3).

Secondly, a high degree of overlap between the double layers associated with the opposing internal walls of the thylakoid would result in a significant negative potential difference throughout the intrathylakoid space in comparison to the external suspending medium. Dark adapted thylakoids would therefore be expected to 'accumulate' divalent cations in preference to monovalent cations and exclude anions from the intrathylakoid space. Initiation of light induced proton pumping on illuminating thylakoids would

result in protonation of the fixed negative charges on the inner surface of the thylakoid and cause divalent cations to efflux from the intrathylakoid space. This provides an explanation for the data of Hind *et al*[100] who found that relatively large amounts of divalent cations effluxed from the intrathylakoid space of broken chloroplasts on illumination whilst monovalent fluxes were small in media containing equal external levels of monovalent and divalent cations. This result is also predicted by a recent analysis of Hope *et al* in terms of light/dark changes in Donnan potential between the intrathylakoid and stromal spaces of intact chloroplasts.[249] The treatment of the intrathylakoid space as a $Donna_n$ phase implies that the net negative charge is evenly distributed in the form of diffusible buffer throughout the internal thylakoid volume.[249] However, there is little evidence that this is the case. The analysis of Hope *et al*[249] would be equivalent to that predicted by Gouy-Chapman theory in the special case of highly overlapping double layers. Under such conditions, little difference would exist between the value of ψ at the membrane surface and at other points within the thylakoid space.

It is apparent from the above discussion that the existence of a net negative charge density on the thylakoid may have a profound influence on many observable properties of both *in vivo* and isolated broken chloroplasts. Chlorophyll *a* fluorescence and membrane stacking of isolated broken chloroplast preparations appears to be affected by the level of cations in the suspending media in a manner which is explicable in terms of the Gouy-Chapman theory of the diffuse double layer. Whether or not cation effects observed in other aspects of photosynthesis research will also be susceptible to quantitative analysis in terms of double layer theory remains a subject for future investigation.

SUMMARY

1. Studies with isolated intact and osmotically shocked broken chloroplasts indicate that fluorescence from *in vivo* chlorophyll *a* is influenced by fixed negative charges on both the outer and inner sufaces of the thylakoid membrane.

2. Slow chlorophyll *a* fluorescence quenching occurs on illuminating isolated intact chloroplasts which appears to involve only the fixed negative charges on the inner side of the thylakoid membrane. The light induced fluorescence lowering is independent of the redox state of the photosystem II traps and can be supported by coupled non-cyclic, or diaminodurene stimulated cyclic electron flow. The fluorescence quenching phenomena are only observed in broken chloroplasts when the suspending media contains low levels of divalent (5 mM) or high levels of monovalent (100 mM) cations to initially create the high fluorescing state. Studies with cation specific ionophores suggest that within the isolated intact chloroplast, the stromal levels of cations (probably Mg^{2+}) are sufficiently high to satisfy this condition. Fluorescence quenching is proposed to occur via membrane conformational changes induced by protonation of fixed negative charges on the inner side of the thylakoid when light dependent proton pumping into the intrathylakoid space is initiated.

3. When high concentrations of diaminodurene (> 12-25 µM) are used to stimulate coupled cyclic electron flow in DCMU treated broken chloroplasts, light induced fluorescence quenching occurs in the presence or absence of sufficient levels of cations in the suspending medium which initially create the high fluorescing state of the thylakoid. Evidence is presented that light induced fluorescence lowering in the presence of high

concentrations of diaminodurene may also involve direct quenching of chlorophyll excited states by the oxidised form of the cofactor in a manner which is enhanced by the lowered intrathylakoid pH of illuminated chloroplasts.

4. In the absence of the light induced high energy state, changes in the fluorescence yield of broken chloroplasts brought about by varying the cation content of the suspending medium appear to involve only fixed negative charges on the outer side of the thylakoid membrane. The high fluorescence yield observed in broken chloroplasts washed and suspended in media containing no added cations appears to be due to the presence of residual divalent catons associated with the membranes. Addition of low levels of monovalent cations (10 mM) results in lowering of fluorescence which can be restored to the high yield on further addition of high levels of monovalent (100 mM) or low levels of divalent (5 mM) cations. These reversible changes in chloroplast fluorescence show little or no dependency on the lipid solubility, ionic size or coordination number of the effective cation, but are strictly dependent on the net positive charge on the ion. It is proposed that in the absence of a ΔpH gradient monovalent and divalent cations may control fluorescence from broken chloroplasts via purely electrostatic interactions with fixed negative charges on the outer thylakoid surface in a manner which can be quantitively described by Gouy-Chapman double layer theory. The results are consistent with the existence of an average net negative charge density of 2.5 $\mu C\ cm^{-2}$ on the outer surface of the thylakoid membrane.

5. Fluorescence lowering in isolated broken chloroplasts suspended in media containing 5 mM Mg^{2+} is also observed on addition of certain polyvalent cations such as La^{3+} and poly-L-lysine. This type of cation

induced fluorescence quenching appears to be largely irreversible and may occur from specific binding of the cation to the thylakoid as a result of the high initial electrostatic attraction exerted by the negatively charged membrane surface. The role of hydration energy is also discussed in the mechanism of irreversible cation induced fluorescence lowering.

6. It is shown that changes in chloroplast fluorescence between low and high yield brought about by light induced high energy state effects, or variations in the monovalent/divalent cation content of the suspending medium of broken chloroplasts, may be due (at least in part) to changes in the distribution of absorbed light energy between photosystem I and photosystem II. However, it is suggested that in in vivo spinach and pea chloroplasts only the magnitude of the high energy state may be involved in the mechanism regulating the relative distribution of light energy to the two photosystems.

7. The existence of fixed negative charges which may give rise to a diffuse double layer at both the inner and outer thylakoid membrane/aqueous interfaces may also have a profound influence on other observable properties of both isolated, and in vivo chloroplasts. Some of these are discussed with particular reference to thylakoid volume and stacking phenomena.

Appendix

I Gouy-Chapman Theory of the Diffuse Double Layer

The distribution of ionic species in a system at equilibrium is such that the mean electrochemical potential of an ion at any point is equal to that at any other point within the system, thus

$$\bar{\mu}_i = \bar{\mu}_{i\infty} \qquad \text{.....Eq. A1}$$

where $\bar{\mu}_i$ is the mean electrochemical potential of species i at a point near the membrane and $\bar{\mu}_{i\infty}$ is the mean electrochemical potential of species at infinite distance from the membrane.

Assuming that the electrochemical potential of ionic species, i, is completely described by the chemical and electrical thermodynamic quantities and neglecting activity coefficients, the distribution of the ith ion at any point xcm from the membrane surface is described by the Boltzmann equation

$$c_i = c_{i\infty} \cdot \exp\ (-ZF\Psi/RT) \qquad \text{.....A2}$$

where c_i is the concentration at a point xcm from the membrane surface

$c_{i\infty}$ is the concentration in the bulk solution (at infinite distance from the membrane surface).

Ψ is the electrical potential difference at point x relative to the bulk solution (where $\Psi = 0$).

F is the Faraday

R is the gas constant

T is the absolute temeperature

Z is the valency (including sign)

Assuming the membrane surface to be infinitely flat and bearing a uniformly spread charge density of q $\mu C\ cm^{-2}$, the electrical potential difference at any point x, relative to the bulk solution is given by the Gauss equation

$$\frac{d\Psi}{dx} = -\frac{4\pi}{\varepsilon} q \qquad \text{..... Eq. A3}$$

where ε is the permittivity of water = $78 \cdot 5.4\pi\ \varepsilon_o$, where ε_o is the permittivity of a vacuum.

For membranes suspended in aqueous solutions at equilibrium, q is balanced by an equal but opposite diffuse charge density in the layer of solution adjacent to the membrane surface, thus:

$$q = \int_{x=0}^{x=\infty} -\rho \qquad \text{..... Eq. A4}$$

where ρ is the space charge density of ions in solution in the plane parallel to the membrane surface at distance xcm from the thylakoid surface. ρ is also given by equation A5.

$$\rho = \sum_i Z_i F c_i \qquad \text{..... Eq. A5}$$

where c_i now includes the mean concentrations of the ith ion at all points in a plane parallel to the membrane and of distance xcm from the membrane.

Combination of equations A3 and A 4 leads to the Poisson equation

$$\frac{d^2\Psi}{dx^2} = -\frac{4\pi}{\varepsilon} \rho \qquad \text{..... Eq. A6}$$

The Poisson equation can be combined with equations A5 and A2 to yield the Poisson-Boltzmann expression:

$$\frac{d^2\Psi}{dx^2} = -\frac{4\pi}{\varepsilon} \sum_i Z_i F c_{i\infty} \exp\left(\frac{-Z_i F\Psi}{RT}\right) \qquad \text{Eq. A7}$$

This equation can be solved by multiplying both sides by 2. $(d\Psi/dx)$ and integrating, noting that as $d\Psi/dx \to 0$, $x \to \infty$ and $\Psi \to 0$.

$$\int_{\frac{d\Psi}{dx}=0}^{\frac{d\Psi}{dx}=\frac{d\Psi}{dx}} 2 \frac{d\Psi}{dx} \cdot \frac{d^2\Psi}{dx^2} = \int_{\Psi=0}^{\Psi=\Psi} -\frac{8\pi}{\varepsilon} \sum_i Z_i F c_{i\infty} \exp\left(\frac{-Z_i F\Psi}{RT}\right) \cdot \frac{d\Psi}{dx}$$

Eq. A8

thus:

$$\left(\frac{d\Psi}{dx}\right)^2 = \frac{8\pi RT}{\varepsilon} \sum_i c_{i\infty} \left[\exp\left(\frac{-Z_i F\Psi}{RT}\right) - 1\right] \qquad \text{Eq. A9}$$

or

$$\left(\frac{d\Psi}{dx}\right) = \pm 2 \left(\frac{2\pi RT}{\varepsilon}\right)^{\frac{1}{2}} \left| \sum_i c_{i\infty} \left\{\exp\left(\frac{-Z_i F\Psi}{RT}\right) - 1\right\} \right|^{\frac{1}{2}} \qquad \text{Eq. A10}$$

The field strength, $(d\Psi/dx)$ can be related at any point with the potential difference at that point (Ψ) relative to the bulk solution. Since the Gauss equation also relates the field strength at the membrane surface $(x = 0)$ to the surface charge density on the membrane q, combination of equations A3 and A10 yields:

$$q = \pm \left[\frac{RT\varepsilon}{2\pi} \sum_i c_{i\infty} \left(\exp \left(\frac{-Z_i F \Psi_0}{RT} \right) - 1 \right) \right]^{\frac{1}{2}} \quad \text{..... Eq. A11}$$

When the membranes are suspended in a medium containing an electrolyte of valency Z - Z such as KCl or $MgSO_4$, equation A11 reduces to:

$$q = 2A (c_\infty)^{\frac{1}{2}} \sinh \left(\frac{ZF\Psi_0}{2RT} \right) \quad \text{.....Eq. A12}$$

where $A = \left(\frac{RT\varepsilon}{2\pi} \right)^{\frac{1}{2}}$

For aqueous solutions at 25°C, substituting appropriate numerical values for R, T, ε, π, and F gives:

$$q = 11.74 (c_\infty)^{\frac{1}{2}} \sinh \left(\frac{Z\Psi_0}{51.7} \right) \quad \text{.....Eq. A13}$$

where q is expressed in $\mu C\ cm^{-2}$, c_∞ in moles l^{-1} and Ψ_0 in mV.

Once equation A13 has been solved for Ψ_0, the local concentration of ions in solution immediately adjacent to the membrane surface can be found by substitution of the value of Ψ_0 into the Boltzmann equation.

II Distribution of Potential in Space

The electrical potential difference, Ψ_0, at the surface declines towards zero with increasing distance, x in a direction perpendicular to the membrane surface. The value of Ψ at any point x can be found on integration of equation A10. In the presence of a single Z - Z electrolyte only, equation A10 becomes:

$$\frac{d\Psi}{dx} = \pm \left(\frac{8\pi\ RT\ c_\infty}{\varepsilon}\right)^{\frac{1}{2}}\ 2 \sin h \left(\frac{ZF\Psi}{2RT}\right) \qquad \text{..... Eq. A14}$$

After multiplying both sides by (ZF/2RT), equation A14 is written for integration thus:

$$\int_{\frac{ZF\Psi_o}{2RT}}^{\frac{ZF\Psi}{2RT}} \frac{d(ZF\Psi/2RT)}{\sin h(ZF\Psi/2RT)} = -\int_o^x \kappa\, dx \qquad \text{..... Eq. A15}$$

where $\kappa = \left(\frac{8\pi\ Z^2F^2c_\infty}{RT\ \varepsilon}\right)^{\frac{1}{2}}$

Integration of equation A15 with boundary conditions $x = 0$, $\Psi = \Psi_o$ yields:

$$\kappa x = \ln\left(\tan h \left(\frac{ZF\Psi_o}{4RT}\right)\right) - \ln\left(\tan h \left(\frac{ZF\Psi}{4RT}\right)\right) \qquad \text{..... Eq. A16}$$

When Ψ_o and c_o are known, the potential at any point x can be computed. The value of Ψ decays non-expotentially with increasing distance x as shown for several values of c_∞ in Fig. 4.17

III Assumptions of Gouy-Chapman Theory

a) The membrane is assumed to be infinitely flat with a fixed negative charge "smeared-out" uniformly over the surface. In practise the membrane can be considered infinitely flat if the radius of the surface area is more than 30-fold greater than the perpendicular distance of the ion from the centre of the membrane. Since the radius of a single

thylakoid is approximately 2,500 Å, whereas the electrical double layer can be considered to have little influence on ions at a distance greater than 100 Å from the surface, this assumption appears to be valid.

b) It is assumed on all integration steps that the dielectric constant term, ε, is independent of Ψ. Grahame(250) has shown that this assumption is valid for a field strength ($d\Psi/dx$) not exceeding 10^6 V cm^{-1}. The maximum field strength occurs at the membrane surface and can be computed from equation A3. It is found that $d\Psi/dx \leqslant 10^6$ V cm^{-1}. when $q \leqslant 7$ μC cm^{-2}, the maximum value of q considered in this thesis.

c) In the Gouy-Chapman treatment of the double layer, ions in solution are considered to be point charges which can approach the membrane to any distance including x = 0. In practise however, the closest plane of approach will be defined by the radius of the hydrated ion, which for small metal cations is of the order 2 - 4 Å. This error may be accounted for by dividing the double layer into two regions, (i) the compact double layer between the charged surface (x = 0) and the plane of closest approach of the ion ($x = x_2$), and (ii) the diffuse double layer which extends out from the plane of closest approach. Thus the potential Ψ_0 ascribed to the point x = 0 in equations A8 - A16 should be redefined as the potential at the plane of closest approach, Ψ_2. However, when q is small (= 2.5 μC cm^{-2}), the difference between Ψ_0 and Ψ_2 is rather small and has for convenience been neglected (see section 4.3 for further discussion on this matter).

IV Application of Gouy-Chapman Theory to Mixed Electrolytes

In order to analyse the antagonistic effects of monovalent and divalent cations on chloroplast fluorescence in terms of their interactions via the diffuse double layer, equation A11 can be expanded to include a mixture of two binary electrolytes of valency $|Z| = 1$ (e.g. KCl) and $|Z| = 2$ (e.g. $MgSO_4$) in bulk solution. Again writing $A = (RT\,\varepsilon/2\pi)^{\frac{1}{2}}$, equation A11 becomes:

$$q = \pm A \left| \sum_i c_{i\infty} \left(\exp\left(\frac{-ZF\Psi_o}{RT}\right) - 1 \right) \right|^{\frac{1}{2}} \qquad \ldots\ldots\ldots \text{Eq. A17}$$

Defining c_∞'' and c_∞' as the bulk concentrations of divalent and monovalent electrolytes respectively, equation A17 becomes, after squaring both sides,

$$\frac{q^2}{A^2} = c_\infty' \left(\exp\left(\frac{-F\Psi_o}{RT}\right) + \exp\left(\frac{F\Psi_o}{RT}\right) - 2 \right) + c_\infty'' \left(\exp\left(\frac{-2F\Psi_o}{RT}\right) + \exp\left(\frac{2F\Psi_o}{RT}\right) - 2 \right)$$

$\ldots\ldots$ Eq. A18

Thus

$$\frac{q^2}{A^2} = 2c_\infty' \left(\cosh\left(\frac{F\Psi_o}{RT}\right) - 1 \right) + 4c_\infty'' \sinh^2\left(\frac{F\Psi_o}{RT}\right) \qquad \ldots\ldots\ldots \text{Eq. A19}$$

Thus

$$\frac{q^2}{A^2} = 2c_\infty' \left(\cosh\left(\frac{F\Psi_o}{RT}\right) - 1 \right) + 4c_\infty'' \left(\cosh^2\left(\frac{F\Psi_o}{RT}\right) - 1 \right) \quad \ldots\ldots \text{Eq. A20}$$

This equation can be rearranged to a quadratic expression after dividing both sides by 2.

$$2c_{\infty}'' \cos h^2 \left(\frac{F\Psi_o}{RT}\right) + c_{\infty}' \cos h \left(\frac{F\Psi_o}{RT}\right) - \left(2c_{\infty}'' + c_{\infty}' + \frac{q^2}{2A^2}\right) = 0 \qquad \text{.. Eq. A21}$$

This expression can be solved for Ψ_o providing that q, c_{∞}'' and c_{∞}' are known. The local concentrations of monovalent (c_o') and divalent (c_o'') cations in solution adjacent to the membrane surface can then be found by substituting the computed value of Ψ_o into the Boltzman equation and solving for each ion individually.

In order to obtain the value of Ψ at points other than the membrane surface in the presence of a mixture of electrolytes of differing valency, equation A10 must be expanded to yield:

$$\frac{d\Psi}{dx} = \pm \left(\frac{8\pi RT}{\varepsilon}\right)^{\frac{1}{2}} \left(4c_{\infty}' \sin h^2 \left(\frac{F\Psi}{2RT}\right) + 4c_{\infty}'' \sin h^2 \left(\frac{F\Psi}{RT}\right)\right)^{\frac{1}{2}}$$

..... Eq. A22

However, this expression cannot be rearranged into a form suitable for integration necessary to solve Ψ as a function of x.

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