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REGULATION OF ELECTRONIC EXCITATION ENERGY DISTRIBUTION IN THE PRIMARY PHOTOPROCESSES OF PHOTOSYNTHESIS IN THYLAKOIDS

University of Illinois at Urbana-Champaign PH.D. 1979

REGULATION OF ELECTRONIC EXCITATION ENERGY DISTRIBUTION IN THE PRIMARY PHOTOPROCESSES OF PHOTOSYNTHESIS IN THYLAKOIDS

BY

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B.S., Illinois Institute of Technology, 1972

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biophysics
in the Graduate College of the
University of Illinois at Urbana-Champaign, 1979

Urbana, Illinois

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN

THE GRADUATE COLLEGE

December, 1978

WE HEREBY RECOMMEND THAT THE THESIS BY

DANIEL WONG

ENTITLED REGULATION OF ELECTRONIC EXCITATION ENERGY DISTRIBUTION IN THE PRIMARY PHOTOPROCESSES OF PHOTOSYNTHESIS IN THYLAKOIDS

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ACKNOWLEDGMENT

I express my deepest appreciation to Professor Govindjee for his guidance and patience. With his broad interest and understanding of photosynthesis, he has in numerable instances steered me away from details along the main path of my research; this I benefited invaluably. I am also thankful to him and his wife, Rajni, for their hospitality during my years in Urbana-Champaign.

I am appreciative of Dr. Marrib Bazzaz for her help earlier in my career, and Dr. Paul Jursinic for helpful discussions. To Dr. Tom Wydrzynski, Miss Rita Khanna, and Mr. Jim Fenton, I am most appreciative of their friendship. I am particularly in debt to Mrs. Carol Fenton for her help in the meticulous typing of the first draft of this thesis.

I thank Dr. Sathamm Saphon for his expert help in the measurement of the absorbance change at 515 nm, to Professor Antony R. Crofts for the use of his spectrophotometer in these measurements, and to Professors Charles J. Arntzen and Colin A. Wraight and Dr. Prasanna Mohanty for helpful comments on my manuscripts. I also thank Dr. Reto J. Strasser for very helpful discussion of the kinetic model for the analysis of low temperature fluorescence data. Particularly, I thank Professor Henri Merkelo for his interest and help in making the fluorescence lifetime measurements possible.

I am appreciative of the financial support through research and teaching assistantships; for these, I thank the National Science Foundation (NSF-Rann Grant GI 31605, and Grant PCM 76-11657) and the Department of Physiology and Biophysics.

Finally, my special thanks to my parents who have made it all possible.

To My Parents

Theoretical insights flourish best when the thinker is apparently wasting time.

J. Robert Oppenheimer

We never know what we are talking about, nor whether what we are saying is true.

Bertrand Russell

When you can measure what you are speaking about, and can express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind.

W. Thomson, Lord Kelvin

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GLOSSARY OF TERMS

A	fraction of photosystem II reaction centers in the "open" state
c	exictation transfer coupling coefficient between the antenna and the reaction center; i.e., the probability that the excitation absorbed by the antenna is transferred to the reaction center
Chl	chlorophyll
Chl a_I, Chl a_{II}	chlorophyll a in the antenna complex of photosystem I, photosystem II
Chl LH	chlorophyll in the light-harvesting chlorophyll a/b protein complex
D	(1) alternate electron donor to the reaction center chlorophyll a of photosystem II (2) as a subscript it denotes the non-radiative dissipation pathway for singlet excited chlorophyll
DAD	2,3,5,6,-Tetramethyl-p-phenylenediamine; also called diaminodurene
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; also called dibromothymoquinone
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DCPIP, $DCPIPH_2$	2,6-Dichlorophenolindophenol; oxidized and reduced forms
DNB	1,3-Dinitrobenzene
E	total absorbed energy
E_I, E_{II}	energy used in photochemistry in photosystem I, photosystem II
E_f	energy dissipated in fluorescence
E_{nr}	energy dissipated by non-radiative means
f	efficiency of excitation energy dissipation by a closed photosystem II reaction center relative to an open one

F	(1) fluorescence intensity (2) subscript notation for fluorescence
F^o	fluorescence intensity in the absence of quenching
F_o	constant or O level fluorescence intensity
F_M	maximum or P level fluorescence intensity
F_{hh}	intensity of horizontally polarized fluorescence using horizontally polarized excitation
F_{hv}	intensity of vertically polarized fluorescence using horizontally polarized excitation
F_{vh}	intensity of horizontally polarized fluorescence using vertically polarized excitation
F_{vv}	intensity of vertically polarized fluorescence using vertically polarized excitation
$F_{i(O)}$, $F_{i(v)}$, $F_{i(M)}$	theoretical amplitudes of constant, variable, and maximum fluorescence from system $\mathit{1}$ and $\mathit{2}$ denoted by i = 1, 2, respectively
$F_{1(\alpha)}$	theoretical amplitude of fluorescence from chlorophyll <u>a</u> in photosystem I excited by photons absorbed by photosystem I
$F_{1(\beta)(O)}$, $F_{1(\beta)(v)}$	theoretical amplitudes of the constant and variable parts of fluorescence from chlorophyll <u>a</u> in photosystem I sensitized by excitation energy from photons absorbed by photosystem II
$F_{2(vib)(O)}$, $F_{2(vib)(M)}$	theoretical amplitudes of the constant and variable yield parts of the vibrational band of chlorophyll <u>a</u> fluorescence from the antenna complex of photosystemII
$F\lambda$	fluorescence intensity at wavelength λ
$F\lambda_{(O)}$, $F\lambda_{(v)}$, $F\lambda_{(M)}$	experimentally measured amplitudes of constant, variable, and maximum fluorescence at wavelength λ = 690 or 730 nm
FWHM	full-width at half-maximum transmission
G	instrumental correction factor; $G \equiv F_{hv}/F_{hh}$

$h\nu$	an excitation photon
$h\nu'$	a photon of delayed light
I	rate of absorption of photons by photochemical apparatus, photons/time
I_1, I_2	photons absorbed per unit time by PS I, PS II
I_{xi}	quanta dissipated by process x = D, F, or P in system *1*, *1'*, *2*, or *2'* denoted, respectively, by i = 1, 1', 2, or 2'
$I_{T(ij)}$	quanta transferred from system *1*, *2*, or *2'* to system *1*, *1'*, *2*, or *2'* denoted, respectively, by i = 1, 2, or 2' and j = 1, 1', 2, or 2'
I.F.	interference filter
J	rate of production of excited state Chl in the generation of delayed light
k_d	rate constant for energy dissipation by a closed reaction center
k_f, k_F	rate constant for fluorescence
k_h	rate constant for the depopulation of the first excited singlet state of chlorophyll *a* by non-radiative processes
k_p, k_{p_o}	rate constant for the depopulation of the first excited singlet state of chlorophyll *a* by photochemistry; k_{p_o} is the maximum value of k_p
k_T	bimolecular rate constant for energy transfer from the antenna to the reaction center
k_{xi}	rate constant for excitation energy dissipation by pathway x = D, F, or P in system *1*, *1'*, *2*, or *2'* denoted by i = 1, 1', 2, 2'
$k_{T(ij)}$	rate constant for excitation energy transfer from system *1*, *2*, or *2'* to system *1*, *1'*, *2*, *2'* denoted by i = 1, 2, 2' and j = 1, 1', 2, 2'
Σk_i	sum of rate constants of all de-excitation pathways in system *1* or *2* denoted by i = 1, 2
L_i	amplitude of component i of delayed light emission

L_s	intensity of delayed light emission at saturation
L(t)	intensity of delayed light emission at time t after an actinic flash
L(n)	intensity of delayed light emission (at a fixed time, e.g., 100 μs) when the excitation flash is n photons cm^{-2} $flash^{-1}$
LHC	light-harvesting chlorophyll a/b protein complex
M	charge accumulator involved in oxygen evolution
MV	1,1'-dimethyl-4,4'-dipyridinium dichloride; also called methyl viologen
n	number of photons per cm^2 per flash
NADP	nicotinamide adenine dinucleotide phosphate
P	(1) subscript notation for primary photochemistry (2) probability that an open reaction center excapes closure in a flash degree of polarization
PS I	photosystem I
PS II	photosystem II
P680, $P680^+$	reaction center chlorophyll of photosystem II; reduced and oxidized forms
Q, Q^-	first stable electron acceptor of photosystem II; oxidized and reduced forms
R	(1) a 2-electron carrier accepting electrons from Q^- (2) the ratio of variable to maximum fluorescence in the chlorophyll fluorescence transient
S(t)	signal (fluorescence plus delayed light) intensity at time t
T	subscript notation for electronic excitation transfer

$[T]_o$, $[T]_t$	maximum concentration and concentration at time t of open traps of photosystem II
Tris	tris(hydroxymethyl)aminomethane
TSF-I, TSF-II	Triton X-100 subchloroplast particles of photosystem I, photosystem II
TSF-IIa	TSF-II without the LHC component
Z, Z^+	physiological electron donor to the reaction center chlorophyll a of photosystem II (P680); reduced and oxidized forms
α	quanta absorbed by PS I as fraction of total quanta absorbed by PS I and PS II
β	equals 1 - α; i.e., fractional contribution by PS II to the total quanta absorbed
η_{xi}	efficiency of excitation energy dissipation by pathway x = D, F, or P in system $\mathcal{1}$, $\mathcal{1}'$, $\mathcal{2}$, or $\mathcal{2}'$ denoted by i = 1, 1', 2, 2'
$\eta_{T(ij)}$	efficiency for excitation energy transfer from system $\mathcal{1}$, $\mathcal{2}$, or $\mathcal{2}'$ to system $\mathcal{1}$, $\mathcal{1}'$, $\mathcal{2}$, or $\mathcal{2}'$ denoted by i = 1, 2, 2' and j = 1, 1', 2, 2'
λ_{exc}	wavelength of excitation
λ_{obs}	wavelength of observation
σ	absorption cross-section of a photosynthetic unit at the wavelength of excitation
σ_m	absorption cross-section of a chlorophyll molecule at the wavelength of excitation
τ	lifetime
τ_i	lifetime of component i
τ_o	fluorescence lifetime of first excited singlet of chlorophyll a in the absence of other de-excitation processes

τ_{Fi}, $\tau_{Fi(O)}$, $\tau_{Fi(M)}$	lifetime of fluorescence from chlorophyll *a* in photosystem I (i = 1) and in photosystem II (i = 2); subscripts O and M denote reaction centers in the open and closed states, respectively
$\phi(F\lambda_{(M)})$	experimentally measured lifetime of chlorophyll *a* fluorescence at λ = 686, 695, or 730 nm, with the reaction centers in the closed state
ϕ_L	quantum yield of delayed light emission
$\phi_{T(21)(O)}$, $\phi_{T(21(v)}$, $\phi_{T(21(M)}$	constant, variable and maximum yields of excitation energy transfer from the antenna complex of photosystem II to that of photosystem I, $\mathcal{1} \rightarrow \mathcal{2}$
Φ_{F_o}	fluorescence yield from analytic flash without prior actinic flash; *i.e.*, O-level fluorescence yield
Φ_{F_M}	P-level fluorescence yield
$\Phi_{F(t)}$	fluorescence yield at time t after actinic flash
Φ_M	maximum value of $\Phi_{F(t)}$ for a particular exciting flash intensity
$\Phi_n(t)$	fluorescence yield at time t normalized to the Φ_{F_o} value for the sample
Φ_{P_o}	quantum yield of primary photochemistry

CHAPTER 1

GENERAL INTRODUCTION

1.1 Photosynthesis

Photosynthesis in green plants involves the oxidation of water to oxygen and the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$), mediated by two photoreactions in series, shown in detail in Fig. 1.1. A brief description is given in the legend of the figure; some of the details shown are still hypothetical. Optimal quantum efficiency of photosynthesis requires the balanced input of light quanta to the reaction centers, labelled as P700 and P680, of the two photosystems; an efficient regulatory mechanism must exist to accomplish this. The details of such a mechanism remain obscure but it appears that the light-induced cation translocation across the thylakoid membrane in chloroplasts may be related to the regulation of energy distribution between the two photosystems (see Barber, 1976; Williams, 1977; Arntzen, 1978).

A brief survey directed at studies closely related to the question of regulation of electronic excitation energy distribution in the two photosystems follows; the emphasis is on some of the early works and the key concepts.

1.2 Cation Effects on Fluorescence and Electron Transport

Homann (1969) first reported that cations increase the variable part (i.e. the part which increases as the reaction centers close; cf. Papageorgiou, 1975) of chlorophyll a flourescence at 685 nm at

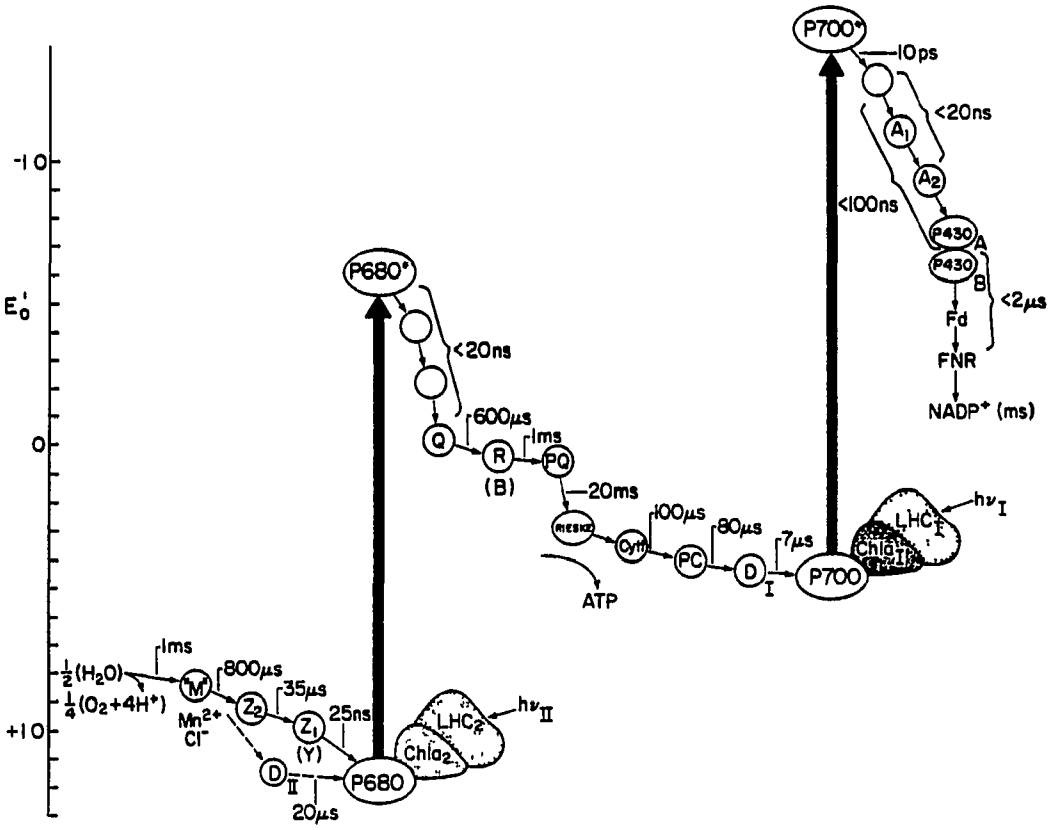

Figure 1.1 The Z-Scheme for Photosynthetic Electron Transport. Excitation (left vertical arrow) of the photosystem II reaction center (P680) results in the removal of an electron from $P680^+$ giving $P680^+$, and reduction of the first stable electron acceptor Q in <20 ns, forming Q^-. The possible participation of intermediates between P680 and Q has not yet been investigated. $P680^+$ is reduced by electrons from water oxidation mediated by the charge accumulator M, of the oxygen evolving apparatus, and electron transfer intermediates Z_2 and Z_1 (or Y), with the approximate half-times indicated with the arrow for each step. Excitation (right vertical arrow) of the photosystem I reaction center (P700) is followed by electron removal from P700 giving $P700^+$ in <10 ps (J. Fenton, M. Pellin, Govindjee, and K. Kaufmann, unpublished). The primary electron acceptor is yet unidentified. However, the electron, after primary charge separation, is transferred to intermediates A_1, A_2, P430 (A and B), ferredoxin, ferredoxin-NADP-reductase (FNR), and finally $NADP^+$. The $P700^+$ is re-reduced by electron transfer from photosystem II through the intersystem electron transport chain as follows: The electron on Q^- passes in sequence through a two-electron acceptor R (or B), a special form of plastoquinone, the plastoquinone pool (PQ), a Rieske protein, cytochrome f, plastocyanin (PC), an intermediate D, and finally $P700^+$. [This figure is taken from a review by Govindjee (1979). Original references to the various electron transport components may be found in the above review.]

room temperature. This phenomenon exists even when net electron flow is inhibited by the addition of 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU). Murata (1969) independently studied this phenomenon and concluded that the chlorophyll a fluorescence yield increase at 685 nm is the result of inhibition of energy transfer ("spillover") from the bulk chlorophyll a in the more fluorescent photosystem II (PS II) to the bulk chlorophyll a in the less fluorescent photosystem I (PS I). This conclusion was based on two lines of consideration: first, the empirical findings that at pH 7.6 divalent cations simultaneously (a) enhanced the rate of electron flow through PS II from water to 2,6-dichlophenol-indophenol ($H_2O \rightarrow$ DCPIP), and depressed the rate of electron flow through PS I from ascorbate-reduced-DCPIP to nicotinamide adenine dinucleotide phosphate ($DCPIPH_2 \rightarrow NADP^+$), and (b) increased the relative intensities of fluorescence from PS II (bands with peaks at ~685 nm and ~695 nm) to that from PS I (at 735 nm) at 77 K, and second, a kinetic analysis of the room temperature fluorescence transient.

In most of the above studies low concentrations of tricine buffer or salts of monovalent cations were present. Gross and Hess (1973) found that the divalent cation-induced fluorescence increases were absent in spinach chloroplasts when tricine and monovalent cations were deleted from the suspending medium. Under such conditions, the addition of low concentrations (2-10 mM) of monovalent cations (K^+, Na^+ and tetraethylammonium) caused a decrease in chlorophyll a fluorescence at room temperature. Fluorescence emission spectra observed at 77 K showed that the decrease in intensity at 685 nm was accompanied by an

increase at 735 nm. These results were suggested to imply that low concentrations of monovalent cations promote "spillover" of excitation energy from PS II to PS I. Subsequent addition of high concentrations of monovalent (~100 mM) or low concentrations of divalent cation ($\lesssim$ 10 mM) caused reversal of the monovalent cation effect (see also VanderMeulen and Govindjee, 1974; Wydrzynski et al., 1975). However, the effects of high concentrations (100-300 mM) of monovalent and low concentrations of divalent cations may not be identical. In addition, monovalent cations give different results under different experimental conditions. Malkin and Siderer (1974) reported that, at pH 7.5, even when a large (2.4 fold) enhancement of the maximum steady-state fluorescence was observed when the NaCl concentration was increased from 10 mM to 100 mM, little or no effect on the oxygen evolution rate (water to ferricyanide Hill reaction) in limiting light was observed. On the other hand, Harnischfeger and Shavit (1974) found an increase in the rate of ferricyanide reduction in limiting light upon increasing the KCl concentration, which they attributed to the increase in ionic strength. Increase of ferricyanide reduction rate by NaCl and KCl has also been observed by Rurainski and Mader (1977). Gross et al. (1976) found that, for thylakoids suspended in 100 mM sucrose + 0.3 mM Tris-HCl (pH 8), a decrease in the rate of electron flow from H_2O to DCPIP occurred when the NaCl concentration was increased, saturating around 10 mM with a half-maximal effect at 2-3 mM NaCl, and a relatively constant effect for concentrations between 10 and 100 mM. However, in 50 mM Na-tricine buffer at pH 8.2, 33 mM KCl caused an increase in the

DCPIP reduction rate.

An alternative to the cation-induced changes in the "spillover" of excitation energy from PS II to PS I is a model that assumes cation-induced changes in the initial distribution of quanta in the two pigment systems. Such a model was developed by Bonaventura and Myers (1969) for light-induced changes in chlorophyll *a* fluorescence in the green algae *Chlorella* (also see Duysens, 1972). Marsho and Kok (1974), who investigated the effects of cations on various aspects of electron transport in spinach chloroplasts, concluded that their data could best be explained by this model in which one assumes cation-induced changes in "α" (the fraction of quanta initially delivered to PS II).

Butler and Kitajima (1975a,b) used a tripartite structural model for the explanation of chlorophyll *a* fluorescence at low temperatures (77 K). In this model, the pigmented portion of the photosynthetic apparatus consists of a photosystem I chlorophyll *a* complex, a photosystem II chlorophyll *a* complex, and a light-harvesting chlorophyll *a*/*b* protein complex (LHC) which contains a large portion of the bulk chlorophyll *a* and all of the chlorophyll *b*; the chlorophylls associated with these complexes are referred to as Chl a_I, Chl a_{II}, and Chl LH, respectively. In Butler and Kitajima's picture, the initial partition of energy between the two photosystems is controlled by LHC, and is described as the fractions, α and β, of absorbed quanta initially distributed to PS I and PS II, respectively. (Note that the β of Butler and Kitajima is the equivalent of the α_{II} of Malkin (1966) and the α of Bonaventura and Myers). In addition, energy can be transferred

from the PS II complex to the PS I complex either directly or indirectly by way of LHC; "spillover" is analysed as a change in the rate constant of energy transfer from PS II to PS I. Thus, Butler and Kitajima's model provides for the study of both the initial energy distribution (α changes) and redistribution ("spillover") between PS II and PS I as independent variables.

Jennings and Forti (1974) suggested, on the other hand, that Mg^{2+} ions alter the efficiencies of fluorescence and non-radiative dissipation processes in favor of fluorescence. Malkin and Siderer (1974) proposed that high-salt medium (100 mM NaCl in their studies) tends to decrease the non-radiative transition constants and, perhaps, also photochemistry. Finally, Rurainski and Hoch (1972) proposed a Mg^{2+}-induced increase in the concentration of PS II reaction centers to explain their observation that Mg^{2+} enhances both the $NADP^{+}$ reduction rate and the chlorophyll a fluorescence intensity. Li (1975) argued that since the lowered variable chlorophyll a fluorescence yield in thylakoids in the absence of magnesium was not increased by increasing the actinic light intensity, the magnesium-depleted thylakoids contained inactive centers. Bose and Arntzen (1978) favored the activation of the PS II reaction centers for the interpretation of their observation of an ~2-fold increase in the steady-state flash yield of oxygen evolution and of oxidation of hydroxylamine by Mg^{2+}, using a Pt/Ag/AgCl rate-electrode.

1.3 Cation Effects on the Structural Changes of Thylakoid Membranes

Light scattering at an angle of 90° and transmission are very useful parameters to follow light-induced "structural" changes in chloroplasts. Extensive research (see Murakami et al., 1975) has been done on the relation of light scattering to the structural state of the thylakoid membranes; light scattering changes seem to reflect more closely a structural, physical state within the membrane, while changes in the transmission reflect volume and/or shape changes. Murata (1971) reported that the divalent cation-induced increase in chlorophyll a fluorescence and the changes in 90° light scattering at 534 nm have similar kinetics. Gross and Prasher (1974) reported a parallel time course of the fluorescence and turbidity (transmission) changes by monovalent cations. All these changes were slow, requiring 2-5 minutes to complete. The cation concentration dependence of fluorescence and light scattering were reported to be the same by both groups of workers, suggesting that structural changes are somehow involved in the cation effects on fluorescence. This suggestion was supported by the observation by Mohanty et al. (1972) and Jennings and Forti (1974) that glutaraldehyde fixation, which blocks structural changes of chloroplast fragments, eliminates the magnesium enhancement of fluorescence yield. However, Mohanty et al. (1972) concluded that there was no direct correlation between structural and fluorescence changes as the kinetics of these changes do not always match. (Bose and Arntzen (1978) have also shown that glutaraldehyde fixation eliminates the Mg^{2+} enhancement of the flash yield of O_2 evolution.) VanderMeulen and Govindjee (1974)

reexamined both the monovalent and divalent cation effects by simultaneously monitoring, under several conditions, chlorophyll a fluorescence at 685 nm and 90° light scattering at 540 nm, and reported a faster time course for Na^+-induced decrease in light scattering than in fluorescence. A close correspondence between fluorescence and light scattering for divalent cations was also absent in several cases. Furthermore, experiments by Prochaska and Gross (1977) on photosystem I (TSF-I) and photosystem II (TSF-II) Triton X-100 subchloroplast particles show widely different concentrations of cations needed to observe a half-maximal effect for fluorescence changes as for light scattering changes. Thus, it seems that although structural changes occur (see also Schooley and Govindjee, 1976), these changes are not directly related to chlorophyll a fluorescence changes.

1.4 Cation Effects on Membrane Components of Thylakoids

Investigations have been conducted to determine the necessity of various biochemically identifiable components of the thylakoid membrane for the observation of cation effects on fluorescence and electron transport. Murata (1971) reported that Mg^{2+} increases the chlorophyll a fluorescence yield in grana fragments which have PS II and PS I activity, but produces no change in the stroma lamellae that have mainly PS I activity. Mohanty et al. (1972) reported the absence of a Mg^{2+} effect in purified PS II particles from maize. These experiments seem to suggest that both photosystems must be present for the occurrence of the divalent cation effects. However, Wydrzynski et al.

(1975) observed an increase in both the 695 nm and 735 nm bands with respect to the 685 nm band in the emission spectra of PS II particles at 77 K suggesting that the presence of PS I is unnecessary for the manifestation of at least one effect of Mg^{2+} on fluorescence. This finding is further supported by the results of Prochaska and Gross (1977) which show that mono- and di-valent cations cause large decreases in chlorophyll a fluorescence in both TSF-II and TSF-I particles from spinach. Other results show that mono- and di-valent cations cause a decrease in the quantum yield of photochemistry in TSF-II particles but cause an increase in TSF-IIa particles which lack the LHC component (Davis et al., 1977); in the P700-chlorophyll a-protein, prepared by the method of Shiozawa et al. (1974), an increase in the quantum yield of electron transport is observed (Gross and Grenier, 1978). Davis et al. (1977) suggested that, in TSF-II particles, cations cause the appearance of quenching units in the LHC portion of the antenna complex which decrease both the fluorescence and photochemistry. Prochaska and Gross (1977) interpreted the inverse relation between fluorescence and quantum yield of PS I photochemistry as a cation facilitation of energy transfer from the antenna chlorophyll a to P700. The above results for TSF-II particles were confirmed by Horton and Croze (1977). To provide a general interpretation for the results from studies on thylakoids and sub-chloroplast particles, Davis et al. (1977) proposed that the cations act at three levels: (1) thylakoids: here, cooperation of both photosystems is required; this effect is absent in sub-chloroplast particles; (2) individual photosystems separated or in situ: this cation effect is

observed both in the thylakoids and sub-chloroplast particles, e.g. cation effects on the quantum yield of PS II electron transport; and (3) isolated particles only: this effect includes ionic strength induced decrease in quantum yield for electron transport in TSF-II particles and cation-induced increase in the quantum yield of electron transport observed in TSF-IIa particles.

Among the above effects, only (1) and (2) seem to be significant for in vivo photosynthesis. For (1), which involves the regulation of energy distribution between the two photosystems, the light harvesting chlorophyll a/b protein (Thornber, 1975), has been shown to be important (see Arntzen, 1978). Gross and Hess (1974) attempted to identify the site of cation action by characterizing the binding sites on the chloroplast membrane. A site with a dissociation constant of 51 ± 8 μM binds divalent cations (Ca^{2+}, Mn^{2+}, and Mg^{2+}); this binding was correlated with divalent cation-induced changes in chlorophyll a fluorescence and changes in membrane structure. Davis and Gross (1975) extended this investigation to the light harvesting chlorophyll a/b protein. The isolated protein bound divalent cations with a dissociation constant of 32 μM. They suggested that the light harvesting pigment protein could be involved in the control of "spillover" processes in the intact membrane. Akoyunoglou and Argyroudi-Akoyunoglou (1970; 1973; 1977) and Arntzen and coworkers (Armond et al., 1976; Arntzen et al., 1977; Davis et al., 1976) have analysed the changes in photochemical properties and contents of pigment proteins of chloroplasts greened in intermittent light. The latter group showed a correlation

between the appearance of the light harvesting pigment protein (Complex II on polyacrylamide gel) and on the onset of the Mg^{2+} regulation of excitation energy distribution in developing membranes. Their subsequent studies using plants grown under different light intensities and the soybean $Y_{11}y_{11}$ mutant, which has deficiencies in the light harvesting chlorophyll a/b protein, have further confirmed the role of the protein in the Mg^{2+}-induced change in fluorescence. Vernotte et al. (1976) found that the Su-su mutant of tobacco contains very small amounts of LHC and shows slight cation-induced changes in F685 or the ratio of F730/F685. Boardman and Thorne (1976), however, found that cation-induced changes in fluorescence were present in a chlorophyll b-less mutant of barley which has deficiencies in the LHC, and concluded that Complex II is not needed for the fluorescence changes. More recently, polypeptide analysis (see Boardman et al., 1978) of this mutant has revealed that some of the LHC polypeptides are present though not the pigmented Complex II, implying that the non-pigmented portion of the LHC still present in the mutant may be responsible for the cation effects. The involvement of the light harvesting complex in cation regulation of excitation energy distribution in chloroplasts has been explicitly confirmed by Burke et al. (1978) and Lieberman et al. (1978).

1.5 Theoretical Studies

Seely (1973a,b) has made calculations on several model systems designed to show the effect of variable transfer rates and transition

dipole orientations on trapping times. The assumptions of his calculations were that energy transfer is a random, stepwise process, and that the rate depends on spectral overlap and dipole orientation in a manner consistent with Förster's inductive resonance theory. He also simulated a model array with a channel for passage of energy between the two photosystems and showed that the manipulation of the orientations of very few (only six) chlorophylls controlled the extent of intersystem transfer, and the distribution of energy between the two traps. These calculations show the possibility for the control of energy distribution between the two photosystems by very small changes in the membrane components.

Barber and Mills (1976) have provided some possible physical insight into the origin of the cation effects on fluorescence by the application of the Gouy-Chapman equation for solutes of mixed electrolytes to suspensions of chloroplasts. Their calculations suggest that fluorescence changes are not correlated with changes in the surface potential per se but may be qualitatively correlated with the total positive charges immediately adjacent to the membrane surface. A review of the inherent assumptions of Gouy-Chapman theory and their implications in studies on thylakoids has been published by Barber et al. (1977).

1.6 Problems with Existing Evidence for Cation Effects

It appears that with this background information it should be possible to pursue the molecular mechanisms of how cations cause their effects on the thylakoids. However, several questions still exist

which must be answered before meaningful quantitative studies can be conducted. Three of these questions are considered here. The first asks whether cations bring about concomitant changes in the different processes which they have been suggested to affect (namely, the initial distribution of excitation to the two photosystems, the redistribution of excitation from PS II to PS I, the rate constants for radiationless transitions, and the activation of reaction center II), the second asks whether the current basis for the "spillover" hypothesis is valid, and the third asks whether Mg^{2+} increases the total number of PS II reaction centers with capacity for undergoing primary photochemistry.

1.6.1 The "Spillover" Hypothesis

Combining the suggestions of Murata (1969) and Gross and Hess (1973), the "spillover" hypothesis may be stated as follows: Monovalent cations enhance and divalent cations supress the "spillover" or redistribution of electronic excitation energy from PS II to PS I. The hypothesis represents an attempt at a unified explanation for the Mg^{2+} enhancement of PS II processes (685 nm fluorescence and steady-state electron flux through PS II, both at room temperature) and an inhibition of PS I processes (ratios of F730/F685 and F730/F695 at 77 K and steady-state electron flux through PS I at room temperature). From a physical point of view the "spillover" hypothesis is an attempt at stating the principle of conservation of energy, taking the efficiencies of photochemistry and fluorescence in PS I and PS II as the complete set of energy parameters. Such an assumption probably represents an oversimplification of the system, and, indeed, has been challenged by

several observations. Firstly, the Mg^{2+} effect on the rate of PS I photochemistry, namely, the rate of reduction of $NADP^{+}$[□] is found to be pH dependent (Bose, 1974; Rurainski and Mader, 1978). At pH < 7.0 Mg^{2+} induces an increase and at pH ≳ 7.5 Mg^{2+} induces a decrease in $NADP^{+}$ reduction. Room temperature fluorescence at 685 nm is increased by Mg^{2+} at all pH's in the range 6 to 8.5 with greater Mg^{2+} enhancement at the higher pH values (Bose, 1974). The "spillover" hypothesis is questioned on the basis that the reciprocal relation between PS I and PS II photochemistry is observed only at pH's ≳ 7.5, while a parallel change in PS I and PS II photochemistry is inferred for pH < 7.0. This last assertion suggests the possibility of parallel changes in PS I and PS II photochemical rates and fluorescence yield and could invalidate the original arguments of Murata for "spillover"; it appears that the Mg^{2+}-induced increase in PS II photochemical rate need not be at the expense of PS I photochemistry, but could result from a decrease in nonradiative events of de-excitation of chlorophyll a singlet states (see Marsho and Kok, 1974; Jennings and Forti, 1974; Malkin and Siderer, 1974). Thus, the general formulation of the principle of conservation of energy in the chloroplast system must include a fourth energy term, that of energy dissipation by non-radiative pathways, and must be written as

$$E = E_I + E_{II} + E_f + E_{nr} \qquad (1.1)$$

□Caution must be exercised in making conclusions from the Mg^{2+} effect on the rate of $NADP^{+}$ reduction because Mg^{2+} has a separate effect (see Harnischfeger and Shavit, 1974) on the electron transport components on the reducing side of PS I, and may confuse the issue in question.

where E is the amount of energy absorbed, E_I and E_{II} are the energies used in PS I and PS II photochemistry, respectively, E_f is the energy dissipated as fluorescence, and E_{nr} is the energy dissipated by non-radiative means. Secondly, Rurainski and Mader (1977) reported that, under conditions when the rate† of turnover of P700 as a function of the Mg^{2+} concentration is not affected, the rate of ferricyanide reduction increases with increasing Mg^{2+} concentration suggesting that PS II activity is increased but PS I activity is unchanged. These observations could again suggest that $(E_I + E_{II})$ in Eq. 1.1 need not be a constant. Thirdly, Bose et al. (1977) have found that with intermittent-light chloroplasts which do not show Mg^{2+} effects on fluorescence, Mg^{2+} still induces an increase in the rate of photochemistry of PS II. This result implies that the fluorescence changes at 685 nm need not accompany changes in PS II photochemical rates. In addition, the evidence usually cited (Murata, 1969) in support of the "spillover" hypothesis appears unsatisfactory in that the experimental data were collected for two very diverse conditions: fluorescence at 77 K and electron transport at room temperature. The kinetic analysis of room temperature fluorescence transient used by Murata (1969) and that for 77 K fluorescence transient by Butler and Kitajima (1975a,b) are deceptive in that the only variable rate constant competing with fluorescence is that for energy transfer from PS II to PS I.

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†In these experiments the full extent of the P700 signal was, perhaps, not measured (see e.g. Rurainski et al., 1971).

1.6.2 The Activation and Inactivation of Reaction Center II

The existing evidence used for suggesting the activation and inactivation of reaction center II by cations (Rurainski and Hoch, 1972; Li, 1975; Bose and Arntzen, 1978) is rather indirect. The occurrence of an activation-inactivation process must be further tested by more direct measurements.

1.7 Purpose and Scope of Present Thesis

The brief review above strongly suggests the existence of numerous effects of cations on thylakoid structure and function. The ultimate aim of research in this area is, therefore, to understand the molecular mechanism of these different cation effects, and their interdependence. This is presently a very formidable problem. The question we asked is a modest one by comparison, but, nevertheless, its answer is essential for further development of the field.

Our interest was to better understand what events in the thylakoid regulate the distribution of excitation to the photosystems. Specifically, we wanted to know which of the proposed processes: initial distribution of absorbed quanta to the two photosystems, redistribution of quanta from PS II to PS I, radiationless dissipation, and reaction center II activation, are present in sucrose-washed thylakoids, and in what proportions? To answer this question we had to establish which processes exist. This required the establishment of a firm basis for the existence of each process. In the study of excitation redistribution, the latter consisted partly of critically testing some of the

existing techniques--for example, (a) whether 77 K relative fluorescence intensity changes reflect changes in the fluorescence yield, and (b) whether the opposite Mg^{2+}-induced changes in the light-limited electron transport rates in PS I and PS II are pH dependent--and, partly of introducing a new way of qualitatively studying energy redistribution by measuring the room temperature fluorescence polarization as function of emission wavelength. In determining whether reaction center activation by Mg^{2+} occurs we obtained new data from the measurement of the initial amplitude of the flash-induced absorbance change at 515 nm, which has been shown to be directly related to the extent of primary charge separation. New means for establishing the occurrence of initial distribution of quanta to the photosystems at room temperature were introduced from (a) the study of the effect of Mg^{2+} on the relative intensity and degree of polarization of fluorescence, and (b) the study of the intensity of delayed light emission (at 100 μs) as a function of the actinic flash intensity. No definitive means of determining radiationless processes is provided. The second part of our question, regarding the relative importance of the processes which exist, required some new development for the method initially presented by Butler and Kitajima (1975a,b). We, thus, developed a method for estimating the initial fraction of quanta distributed to PS I (α) by the simultaneous evaluation of 77 K fluorescence transient and lifetime data. The relative importance of each existing process was estimated from these low temperature fluorescence measurements, and confirmed by the relative cation-induced changes from room temperature measurements.

The materials and methods used are described in Chapter 2.

In Chapter 3 we present the first parallel measurements of the cation effects on the 77 K chlorophyll a fluorescence lifetimes (τ) at 686, 695, and 730 nm and transients at 690 and 730 nm. The new method of utilizing parallel lifetime and transient measurements from a single sample to calculate α is presented. We are able to conclude that the cation effects (in decreasing order of importance) are: the redistribution ("spillover") of excitation from PS II to PS I, the initial distribution of absorbed quanta to PS II, and some other non-radiative processes in the antennas of PS I and PS II.

In Chapter 4, we show further confirmation of the above conclusions at room temperature from measurements of the cation effects on delayed light emission and fluorescence yield in the μs time scale. This chapter also provides the important conclusion that in our preparations Mg^{2+} does not activate the reaction centers of PS II per se, based on the finding that the initial amplitude of the flash-induced absorbance change at 515 nm is unaffected by Mg^{2+} addition.

In Chapter 5, we provide the most definitive evidence to date in support of the "spillover" hypothesis at room temperature, from the first measurements on the fluorescence polarization changes at 686, 712, 730, and 762 nm with cation addition. From measurements on the excitation spectra for the Mg^{2+}-induced increase in chlorophyll a fluorescence and decrease in polarization of fluorescence at 762 nm, we also provide the first direct evidence for the Mg^{2+} enhancement of energy transfer from Chl a and Chl b in the light-harvesting Chl a/b protein complex to Chl a in the antenna complex of PS II.

In Chapter 6 we present our data on the pH dependence of the phenomenon under investigation: cation effects on the fluorescence transients and lifetimes at room temperature and 77 K, the fluorescence polarization, and rates of PS I and PS II electron transport (at high and low light) at room temperature. It is concluded here that the Mg^{2+} effects on fluorescence and electron transport operate over the pH range 5.5 - 8.5 while the Na^{+} effects (opposite to those of Mg^{2+}) occur over a narrower range around neutral pH.

Finally, Chapter 7 provides a general summary of the major conclusions obtained in the present thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chloroplast and Sample Preparation

Pea (Pisum sativum var. Progress No. 9) seedlings were grown under cool white fluorescent light (16 h photoperiod) in vermiculite and healthy leaves were picked after 15-20 days of growth. Broken chloroplasts were prepared by a modified method of Gross (1971). The leaves were homogenized for 10 s with a Waring blender in 350 mM sucrose buffered to pH 7.8 with 50 mM Tris-HCl. The slurry was filtered through 4 and 12 layers of cheesecloth and centrifuged at 500 x g for 1 min. The supernatant was then centrifuged at 6,000 x g for 10 min. The chloroplasts were osmotically shocked by resuspending the pellet in 100 mM sucrose solution and allowing the suspension to stand for 10 min. at 4°C before recentrifugation at 8,000 x g for 10 min. This salt depletion procedure (i.e., washing with sucrose) was repeated twice and the final "loose" pellet was resuspended in 100 mM buffered sucrose to a chlorophyll concentration of 1.0-2.0 mg/ml and frozen in 0.5 ml aliquots at 77 K. Chlorophyll concentration was determined by the method of Arnon (1949). In each series of experiments, the concentration of the sample (thylakoids) was adjusted to give equal concentrations of chlorophyll in all samples. In each experiment a volume of concentrated stock thylakoid suspension was diluted with 100 mM sucrose + 0.4-2.0 mM Tris-HCl at an appropriate pH from which 3 ml aliquots were taken and small equal volumes of water and/or 1 M

salt solutions were added to give the salt-depleted (control), Na^+ and Na^++Mg^{2+} containing samples. For certain experiments a part of the dilution medium was substituted by 90:10 glycerol-water.

It is of practical interest to note that storage of the thylakoids at 77 K, as described above, did not seem to have any major adverse consequences on fluorescence and electron transport even for a period of about a year. The same cation effects on fluorescence changes were observed in both frozen and freshly isolated thylakoids. Steady-state oxygen evolution rates in frozen-stored thylakoids showed the same rates (~150 μmoles O_2/mg Chl/hr at pH 7) as obtained for freshly isolated thylakoids. A recent report by Volger et al. (1978) shows that thylakoid damage by protein release is minimal when the suspension medium is sucrose.

2.2 Absorption Spectra

The absorption spectra of the thylakoids show peaks at 435 (chlorophyll a), 468 (chlorophyll b + carotenoids), a shoulder at 483 (chlorophyll b + carotenoids), and a peak at 678 nm (chlorophyll a) (Fig. 2.1). The measurements were done with a Cary 14 spectrophotometer with the samples placed close to the photodetector to minimize the effects of scattering. A baseline was recorded between 400 and 750 nm for a water blank, after which the optical densities of the three samples (salt-depleted, Na^+, and Na^++Mg^{2+}) were measured without further adjustment. For a sample containing 5 μg Chl/ml the measured optical density at 750 nm for the three samples in the order given above were 0.02-0.03,

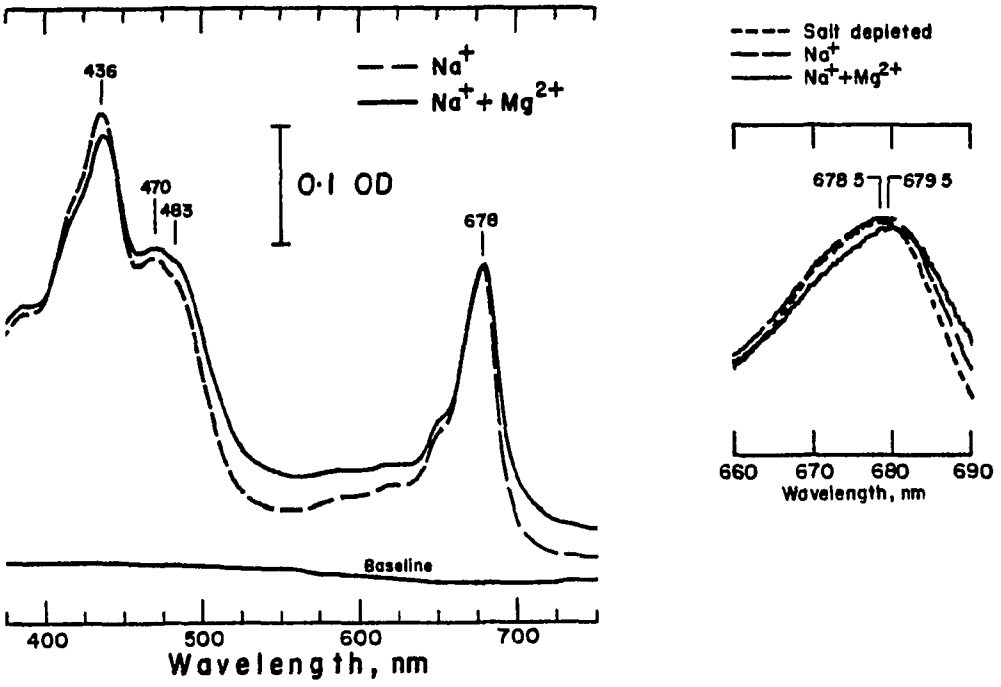


Figure 2.1 Absorption Spectra of Thylakoid Suspensions. Samples in 100 mM sucrose + 2 mM Tris-HCl, pH 7.6; [Chl] = 5 µg/ml. Cations were added as chloride salts to a final concentration of 9.8 mM. Measurements were made with suspensions in 1 cm path-length cuvette placed close to the photomultiplier on a Cary 14 recording spectrophotometer. Temperature = 23° C.

0.03-0.04, and 0.05-0.06, respectively. Typical spectra for the Na^+ and Na^++Mg^{2+} samples are shown in Fig. 2.1. The presence of ~40% glycerol by volume decreased the measured optical density of the sample at 750 nm by 0.03 (i.e., decreased the scattering) implicating the cause to be the mismatch between the refractive index of the thylakoid and the suspension medium. However, a part (~half) of the measured absorbance at 750 nm in the Mg^{2+}-containing sample may be a consequence of increased scattering and possibly the sieve effect (Rabinowitch, 1951, Chapter 22; Duysens, 1956) resulting from more extensive stacking of the thylakoid membranes in this sample over those without Mg^{2+}. This could increase the selective scattering (see Latimer, 1959) in these membranes leading to the 1 nm red-shift of the long wavelength absorption peak to ~679 as compared to ~678 in the other two cases (salt-depleted and Na^+).

2.3 Emission Spectra

The room temperature and 77 K fluorescence emission spectra were recorded with the spectrometer described by Shimony et al. (1967). The readings on the wavelength drum of the monochromator were calibrated to an accuracy of better than 0.2 nm with a standard mercury calibration lamp. For room temperature measurements, 3 ml aliquots of thylakoid suspensions ([Chl]~5 μg/ml) were placed in a Dewar flask furnished with a flat optically clear bottom through which the fluorescence was excited and measured. For 77 K measurements, 0.5 ml aliquots ([Chl]~20 μg/ml) were adsorbed onto two layers of cheese-cloth, held down in the Dewar

flask by a teflon ring prior to freezing. Excitation was by a collimated beam from a Radiant Lamp Corporation (Newark, New Jersey) DDY 750W projection lamp through 5 cm of water filter and an interference filter with maximum transmission at 636 nm (Full-Width at Half-Maximum transmission, FWHM = 8 nm). The excitation irradiance was 2.3 mW/cm^2. The front surface fluorescence was detected by an EMI 9558 B photomultiplier (S-20 photocathode response) through a Schott RG 665 (3 mm thickness; 10% transmission at 655.7 nm, 50% transmission at 664.5 nm, and 80% transmission at 676 nm) glass cut-off filter and a Bausch and Lomb monochromator (model 33-48-45--0.5 meter; 600 grooves/mm; blazed at 750 nm; linear dispersion 3.3 nm/mm slit-width). The slit-width was 1.5 mm for room temperature measurements and 0.6 mm for 77 K measurements. Each spectrum was scanned at 100 nm/90 s; the photomultiplier voltage was amplified, filtered by a low-pass filter with a time-constant of 5 ms, and displayed on an Esterline Angus (Model E 1011s) stripchart recorder at a chart speed of 4 inches/min. The wavelengths were marked using a DC voltage source connected in parallel to the fluorescence signal input to the recorder. The resulting spectrum had 10 nm markings every 0.6 inch. The relative signals were measured at intervals of 2.5 nm (0.15 inch of chart, with a precision of 0.01 inch). In any measurement, the corresponding stray light spectrum was measured under identical conditions upon replacing the thylakoid suspension by a buffer suspension of $BaSO_4$ adjusted to the same optical density as the thylakoid suspension at 750 nm. By subtracting the stray light spectrum from the spectrum recorded with the thylakoid suspension, the uncorrected

fluorescence spectrum was obtained. This spectrum was then corrected for the RG 665 transmission profile, measured with a Cary 14 recording spectrometer, and further corrected for monochromator transmission characteristics and photomultiplier sensitivity to produce the final corrected fluorescence spectrum.

2.4 Fluorescence Transients

When intact algal cells are dark-adapted for 5-10 minutes at room temperature, subsequent exposure to light gives the following fluorescence transient: an initial (O) level, rising rapidly to an intermediate (I) level, declining sometimes slightly to a dip (D), and finally rising to a maximum (P) level (Munday and Govindjee, 1969; Lavorel, 1959). Fluorescence transients of thylakoids show the same pattern except that there is only a slight plateau at the I level, i.e. a dip is never observed (see e.g. Malkin and Kok, 1966). Typical fluorescence transients for sucrose-washed thylakoids suspended in slightly buffered (2 mM Tris) low osmotic strength medium (100 mM sucrose) to which 10 mM NaCl and 10 mM NaCl + 10 mM $MgCl_2$ were added are shown in Fig. 2.2. The lower trace in each transient marks the O level (details of measurement techniques are described below). The main difference between the two transients is in the variable (P-O) fluorescence. If the thylakoids are frozen in liquid nitrogen (77 K) after dark adaptation, the fluorescence transient shows a single-phase rise from O to P (cf. Butler and Kitajima, 1975a).

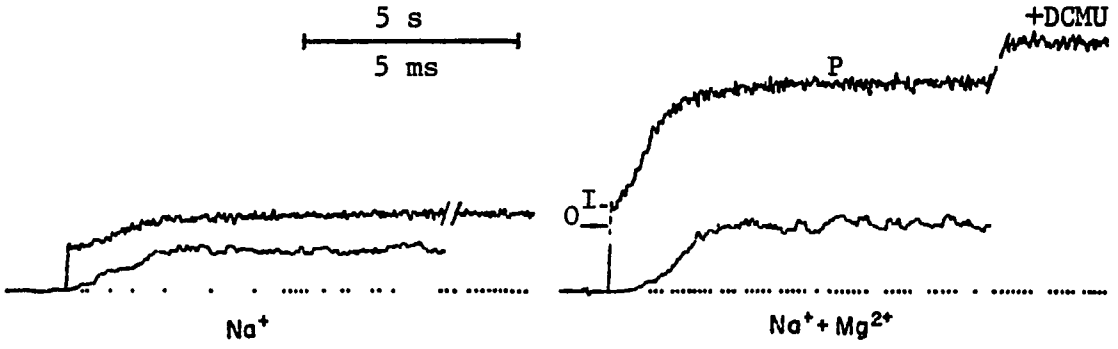


Figure 2.2 296 K Chlorophyll a Fluorescence Transients. Lower traces were for O level fluorescence and correspond to the 5 ms time scale. Upper traces 5 s time scale; the right side shows the P level after addition of 3.3 μM DCMU. [Chl] = 50 μg/ml; $[Na^+] = [Mg^{2+}] = 9.8$ mM; pH = 7.8.

The fluorescence transients were measured with the spectrometer described by Shimony et al. (1967) as modified for transient measurements by Munday and Govindjee (1969). The same procedure as used for the preparation of the samples for spectral measurements were used here. The chlorophyll concentrations used were between 25 and 50 μg/ml. In these measurements the photomultiplier voltages were directly recorded with a Biomation Waveform Recorder (Model 805). The aperture of the shutter for the actinic beam was reduced to give an opening time of <2 ms. For O level measurements the shutter was set to give the sample a 100 ms light exposure, and the Biomation transient recorder was operated in the "pretriggered" mode for a delayed recording of 1800 sampling intervals of 5 μs each--giving 9 ms of recorded transient. The initial rise from the baseline for the O level measurement in Fig. 2.2 is the opening time of the shutter. For P level measurements, the shutter was set for manual control (T stop) and the transient recorder was set at a sampling rate of 5 ms/point. The transients were displayed either on an Esterline Angus (Model E 1011s) stripchart recorder or a Moseley (Model 2D-2) X-Y recorder. All room temperature transients were measured at 685 nm with a band-pass of 6.6 nm or 9.9 nm. Low temperature transients were measured at 690 and 730 nm with the wider band-pass. Stray light signals were determined and subtracted as described in the previous section.

2.5 Cation Effects on P Level Fluorescence at Room Temperature

It has now been well established (cf. reviews by Barber, 1976; Williams, 1977) that the fluorescence changes in thylakoids upon salt

addition are due to the cationic species. To verify this occurrence in our thylakoid preparations we measured the changes in fluorescence at 685 nm (F685) upon addition of various salts containing mono- and divalent ionic species; new inclusions are two non-physiological anions, molybdate and thiocyanate. Our results are presented in Table 2.1. It is clearly seen that, independent of the anionic species, F685 shows a decrease of as much as 40% when the cationic species is monovalent (cf. Gross and Hess, 1973), but shows an increase (~2 fold) when the cationic species is divalent (Homann, 1969). With the confirmation in this important control experiment, we studied the mono- and di-valent cation effects almost exclusively with NaCl and $MgCl_2$. It is important to note that two exceptions to the divalent cation effect on F685 are known, namely Pb^{2+} and Cd^{2+}--these cations cause quenching of fluorescence (Bazzaz and Govindjee, 1974a,b). These quenching effects, initially found in maize chloroplasts, also exist in these preparations from pea chloroplasts: addition of ~10 mM $Pb(NO_3)_2$ to salt-depleted thylakoids at pH ~6.2 gives a 15% inhibition of F685 (Table 2.2). Similarly the trivalent cation Cr^{3+} causes inhibition of fluorescence.

Since it has previously been reported that the Mg^{2+} enhancement of fluorescence is eliminated by the presence of 60% glycerol (Butler and Kitajima, 1975a), the cation effects as a function of glycerol content was investigated. In these experiments, the thylakoid suspensions had a chlorophyll concentration of 100 µg/ml, separate aliquots were taken and incubated with 50 mM NaCl or 50 mM NaCl + 50 mM $MgCl_2$.

TABLE 2.1

Monovalent and Divalent Cation Effects on 685 nm Fluorescence at 23° C

Treatment	Relative Fluorescence (F) Intensities		$F(M^+ + M^{2+})/F(M^+)$	
	Sample 1	Sample 2	Sample 1	Sample 2
Salt-depleted	100	100		
NaCl	90	64		
Na_2MoO_4	81	62		
NaSCN	80	61		
KNO_3	83	62		
NH_4Cl	83	64		
NaCl + $CaCl_2$	193	147	2.15	2.28
Na_2MoO_4 + $CaCl_2$	176	142	2.19	2.29
NaSCN + $CaCl_2$	156	129	1.96	2.10
KNO_3 + $CaCl_2$	193	148	2.34	2.38
NH_4Cl + $CaCl_2$	176	151	2.13	2.37
NaCl + $MgCl_2$	201	160	2.25	2.49
Na_2MoO_4 + $MgCl_2$	169	158	2.09	2.55
NaSCN + $MgCl_2$	159	130	1.99	2.13
KNO_3 + $MgCl_2$	183	149	2.21	2.40
NH_4Cl + $MgCl_2$	181	149	2.18	2.33
$CaCl_2$		158		
$MgCl_2$		173		

Fluorescence excitation at 636 nm (FWHM, 8 nm) and detected through a monochromator (band-pass, 6.6 nm). 3 ml thylakoid suspensions in 100 mM sucrose + 0.5 mM Tris-HCl; final pH 7.3 ± 0.1; [Chl] = 5 µg/ml; [DCMU] = 3.3 µM. M^+ and M^{2+} denote mono- and di-valent cations; $[M^+] = [M^{2+}] = 9.8$ mM.

TABLE 2.2

Effects of Pb^{2+} and Cr^{3+} on 685 nm Fluorescence at 23° C

Treatment	Relative Fluorescence
Salt-depleted	100
9.7 mM $Pb(NO_3)_2$	85
1.0 μM $Cr(NO_3)_3$	94
97.2 μM $Cr(NO_3)_3$	83
2.4 mM $Cr(NO_3)_3$	75

Sample pH = 6.2. Other details as given in the legend of Table 2.1.

After about half an hour, aliquots of these suspensions were diluted 10 times with buffer and 90% glycerol in water (v/v) to give the required glycerol content. The results in Fig. 2.3 show that the monovalent cation effect was unaffected by glycerol up to ~60% glycerol (v/v). At higher concentrations of glycerol the Na^+ effect is diminished. The Mg^{2+} effect decreased by ~35% (from ~2.7 to ~1.7 fold enhancement of F685) when the glycerol content reached 30% (v/v) but remained relatively constant up to ~70%. Above 70% glycerol (v/v) a steep decline of the Mg^{2+} effect was observed with complete impairment at ~80%. Thus, glycerol was not used in most of our studies.

2.6 Microsecond Delayed Light Emission and Fluorescence Measurements

The apparatus for measuring delayed light emission and fluorescence yield rise and decay kinetics in the μs time scale has been described by Jursinic et al. (1977) and in detail by Jursinic (1977). The actinic light source for all measurements was an AVCO Everett Model C102 nitrogen laser (λ emission, 337.1 nm; pulse width at half-maximum, 10 ns). The fluorescence yield rise and decay kinetics were measured by a method similar to that of Mauzerall (1972). An actinic flash was given followed by a very weak probe flash (General Radio Strobotac 1538-A; two Corning CS 4-96 filters [thickness, 5 mm each] and appropriate neutral density filters) given after various delays; the fluorescence intensity in the weak flash was proportional to the fluorescence yield (assuming constant absorption). Delayed light intensities were directly recorded 6-100 μs after an actinic flash; details are given by Jursinic and Govindjee (1977) and Jursinic (1977).

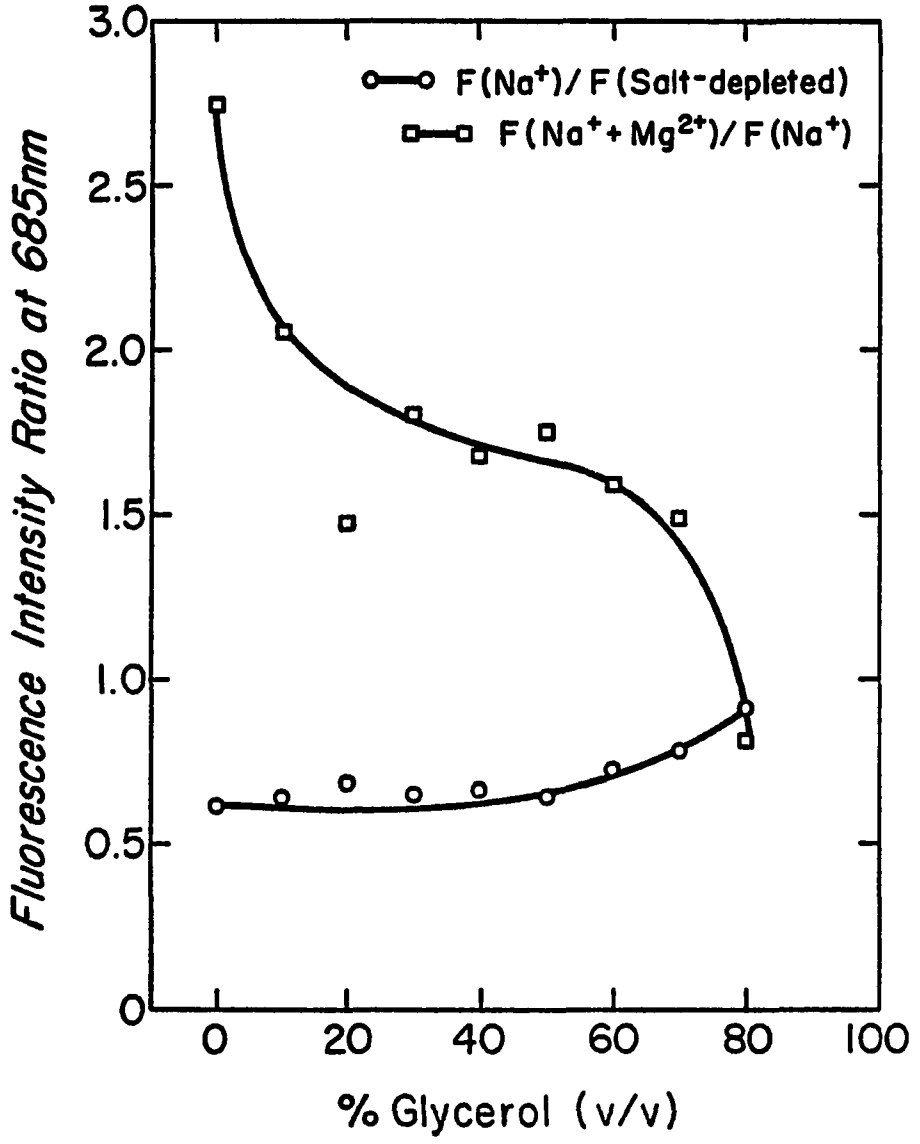


Figure 2.3 Cation Effects on Fluorescence Intensity as Function of Glycerol Concentration. Thylakoid suspensions ([Chl] = 10 µg/ml) prepared as described in text using 100 mM sucrose + 0.5 mM Tris-HCl, pH 8.0 and appropriate volumes of 90:10 solution of glycerol:water. Fluorescence was excited with broad-band blue light (white light through Corning CS 3-73 + CS 4-72 glass filters), and detected through a monochromator (Band-pass, 6.6 nm). Temperature = 21° C.

Both fluorescence and delayed light were detected with an EMI 9558 B photomultiplier through a Corning CS 2-64 and a Schott RG-8 filter combination. Neutral density filters were used where needed. The detection system can be gated on in ~2 μs (Jursinic, 1977).

2.7 Lifetime of Fluorescence

Fluorescence lifetimes were measured by the phase-delay method as described by Merkelo et al (1969). A schematic of the apparatus used in this study is given in Fig. 2.4. Sample excitation was with a He-Ne laser (Spectra Physics Model 125) modified by the inclusion of an intracavity acousto-optic cell driven by a radio frequency broadband (500 kHz-250 MHz) amplifier and a VHF oscillator (Hewlett Packard Model 3200B) at 37.5 MHz, providing mode-locking at 75 MHz. Part of the laser beam (λ = 632.8 nm) was diverted by a beam-splitter to a partial mirror where the transmitted beam was detected by a photomultiplier (RCA 931A) which provided the reference signal to the phase sensitive detector (Hewlett Packard 8405 A Vector Voltmeter); the reflected beam was incident on a PIN-photodiode and provided a continuous monitor of the mode-locking of the laser. The main beam through the beam-splitter was incident on a sample at an angle (as shown) and the fluorescence detected by another photomultiplier (RCA 7102, S-1 photocathode response) placed at an acute angle to the incident beam to avoid the reflection off the cuvette surface. Alternatively, the actinic beam may be incident on the sample along the cuvette face nearest the photomultiplier placed at right angles to the direction of incidence of the

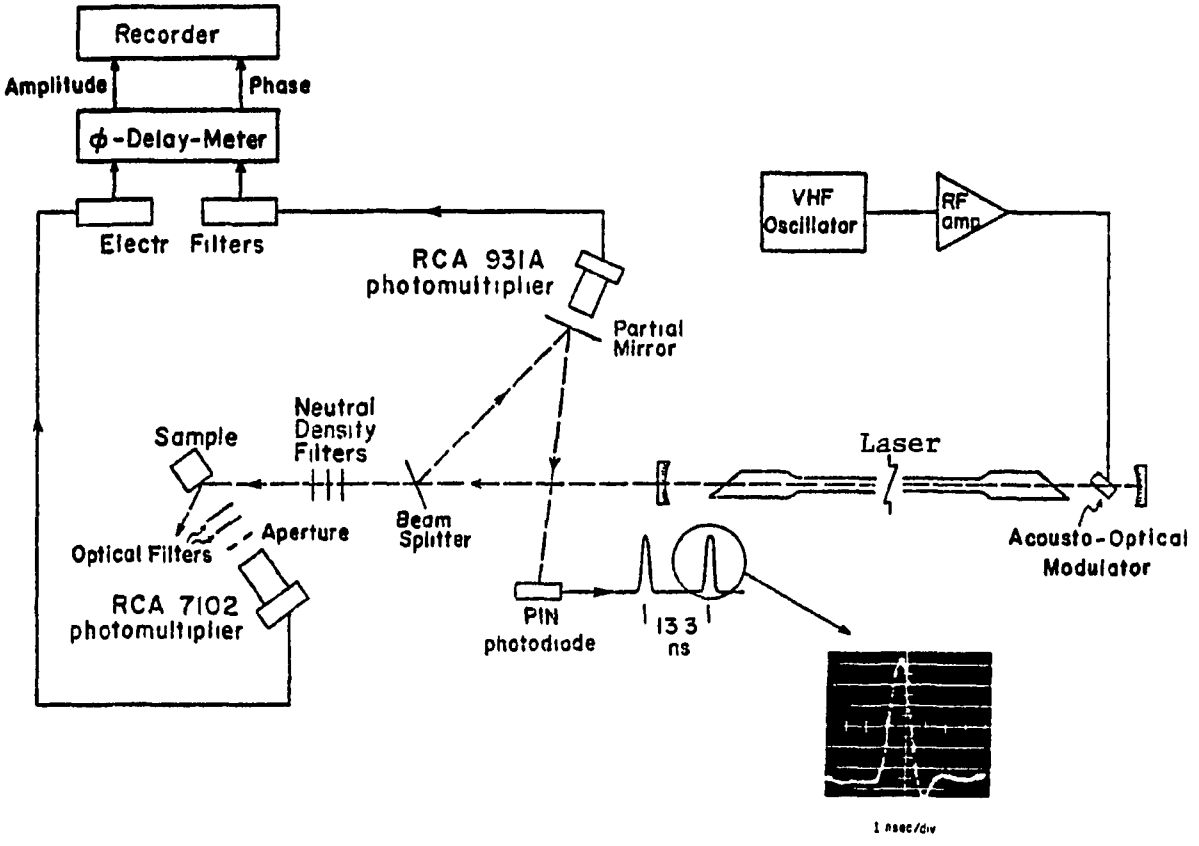


Figure 2.4 Schematic Diagram of the Experimental Setup for the Measurement of Fluorescence Lifetime.

beam. The maximum average irradiance on the sample was 40 mW/cm^2.

A typical lifetime measurement was done as follows: The sample in a 1 cm x 1 cm cuvette was placed in the beam and the fluorescence amplitude and phase recorded through appropriate optical filters (3 mm Schott RG-5 and an interference filter at 686 nm for fluorescence at 686 nm). The sample was then replaced by a freshly prepared suspension of $BaSO_4$ in water and the incident beam attenuated by a neutral density filter to give a signal identical in amplitude to that of the fluorescence. The phase difference between the fluorescence and the scattered light gave the phase-delay ($\Delta\phi$) of the fluorescence emission. The lifetime was then calculated from the relation $\tau = \tan \Delta\phi/2\pi f$ (cf. Bailey and Rollefson, 1953), where $\Delta\phi$ = phase-delay, and f = modulation frequency. For 77 K measurements, the sample was frozen in a 1 mm cuvette and the fluorescence measured from the front surface at the bottom of a Dewar flask furnished with a flat optically clear window. A comparative study showed identical phase for the measured irradiance from both the frozen $BaSO_4$ and the thylakoid suspension, showing that fluorescence represented no significant contribution to the total sample irradiance when the frozen thylakoid suspension was used also as the scattering source. This phenomenon did not hold for room temperature suspensions.

2.8 Degree of Polarization of Fluorescence

A schematic of the apparatus for the measurement of fluorescence polarization is given in Figure 2.5. This instrument was assembled by

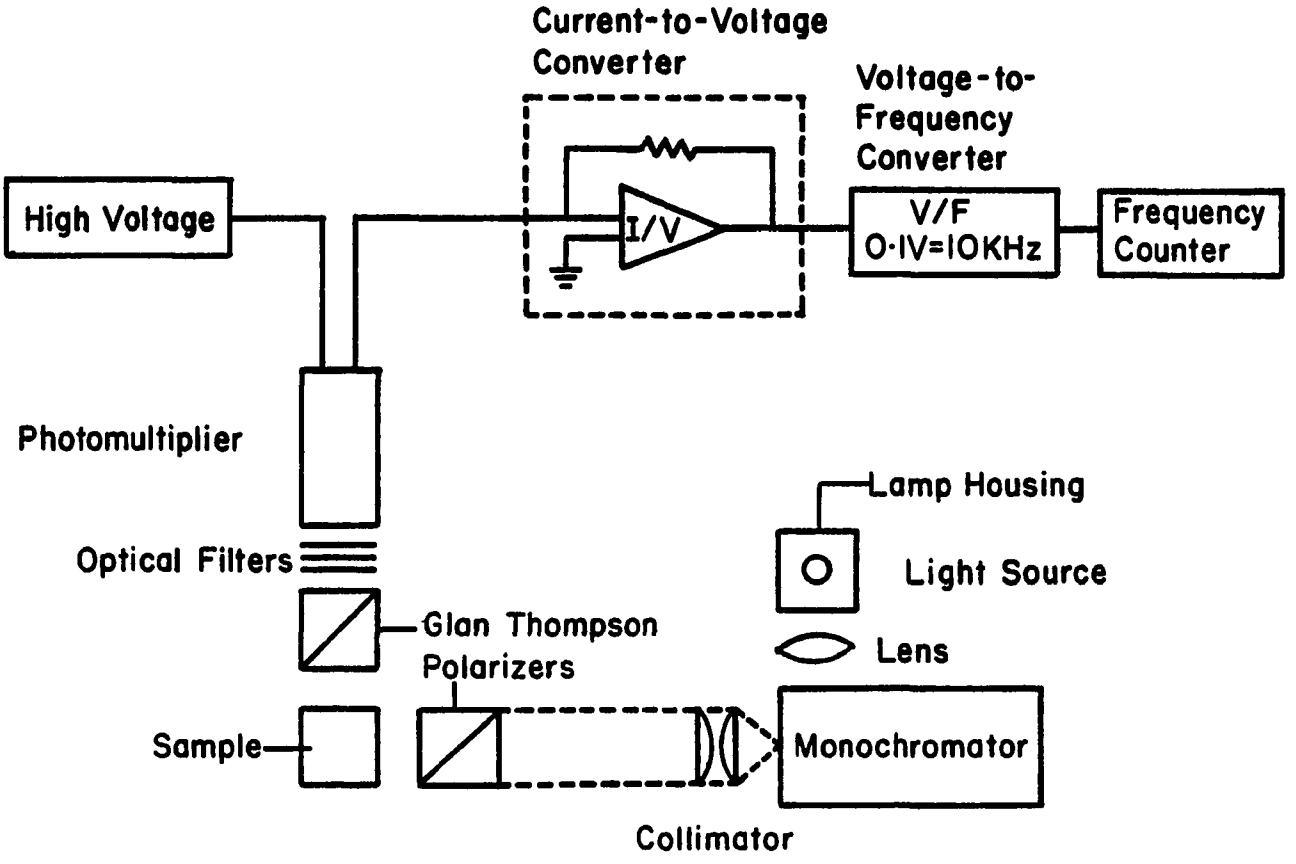


Figure 2.5 Schematic Diagram of Fluorescence Polarization Spectrometer.

the author from parts available from the earlier instrument of Mar and Govindjee (1972). Light from a quartz-iodine lamp (200 W, GE A6.6 AT4/CL) was focused on the entrance slit of a Bausch and Lomb grating monochromator (Model 33-86-45--0.5 meter; 600 grooves/mm; linear dispersion, 3.3 nm/mm); the monochromatic beam was then collimated with a lens system and passed through a Glan-Thompson polarizer (12 x 12 x 24 mm; Karl Lambrecht Corp., Chicago, Illinois). The fluorescence from the sample was passed through a second Glan-Thompson polarizer and appropriate optical filters, and detected by an S-20 photomultiplier (EMI 9558 B). The anode current (I) of the photomultiplier was converted into voltage (V), using an operational amplifier, then to frequency (F), using a voltage to frequency converter (Anadex Instruments Inc., Model DF-110R--2 V, 2 μs spikes output), and the digital signal was displayed on a frequency counter (shop built to the specifications of the Hewlett Packard, Model 5382A except with a threshold of 1.5 V instead of 50 mV) with a gating time of 10 s.

The emission detection system showed a slight systematic inequality in its sensitivities to vertically and horizontally polarized light. The usual practice in this situation is to apply to the horizontally polarized fluorescence a multiplicative correction factor which empirically equalizes the sensitivities of response of the system to the two orientations of polarization. This instrumental correction factor is obtained as the quotient (G) of the vertically (F_{hv}) to the horizontally (F_{hh}) polarized components of fluorescence ($G \equiv F_{hv}/F_{hh}$) when a 10^{-7} M solution of rhodamine B is excited with horizontally

polarized light at 546 ± 1.7 nm; the fluorescence is detected through a Corning CS 3-66 glass filter. The degree of polarization of fluorescence is given by the relation:

$$\underline{P} = \frac{F_{vv} - G \cdot F_{vh}}{F_{vv} + G \cdot F_{vh}} \qquad (2.1)$$

where, F_{vv} and F_{vh} are the vertically and horizontally polarized components of fluorescence from the sample, using vertically polarized excitation.

The instrumental correction factor has been measured for 10^{-7} M rhodamine B in water, 10^{-7} M rhodamine 6G and 10^{-6} M eosin Y in glycerol and in water, and chlorophyll *a* in acetone. In all instances the same value of G was obtained, showing that it is an instrumental property. The results for chlorophyll serve also to verify that in the present instrument the G factor obtained in the region of rhodamine fluorescence is valid also in the region of chlorophyll fluorescence. Thus, 10^{-7} M rhodamine B in glycerol is routinely used for this purpose. It must be cautioned that when thylakoid suspensions are excited with horizontally polarized light, a small negative degree of polarization of fluorescence is observed (see also Whitmarsh and Levine, 1974) and, therefore, thylakoid suspensions are unsuitable for use in determining the instrumental correction factor as done earlier by Mar and Govindjee (1972).

Another crucial instrumental test is the linearity of response of the system to light intensity, particularly the constancy of the

relative sensitivities of response to vertically and horizontally polarized light (cf. Ayres et al., 1974). The latter has been verified (Fig. 2.6) over a > 3 orders of magnitude variation (induced by attenuation of the actinic beam by neutral density filters) in the fluorescence intensity from a 10^{-7} M rhodamine B solution in glycerol, covering a range of intensities above and below those in the measurements of chlorophyll a fluorescence in thylakoid suspensions.

The proper alignment of the polarizers critical for obtaining accurate values of fluorescence polarization was regularly checked. The following criterion was used as an indicator for the satisfactory performance of the instrument in every experiment: When excited with vertically polarized light, the degree of polarization of the 10^{-7} M rhodamine B solution at room temperature (22-23°C) must be ≥ 0.4 (cf. Weber, 1956; Wampler and DeSa, 1974).

2.9 Absorption Changes at 515 nm

Changes of absorption at 515 nm were measured with a single-beam spectrophotometer linked to a transient recorder (Data Laboratory DL905) and a minicomputer (Digital Equipment Corp., PDP 11/10) as described by Saphon and Crofts (1977). The signal-to-noise ratio was improved by averaging 8 or 32 traces using an appropriate program. To ensure a fresh aliquot for every measurement the simple stop-flow system described by Saphon and Crofts (1977) was used. It must be noted that the sample aliquots were not fully dark-adapted in the sense that the low intensity measuring beam (irradiance ~10 $\mu W/cm^2$)

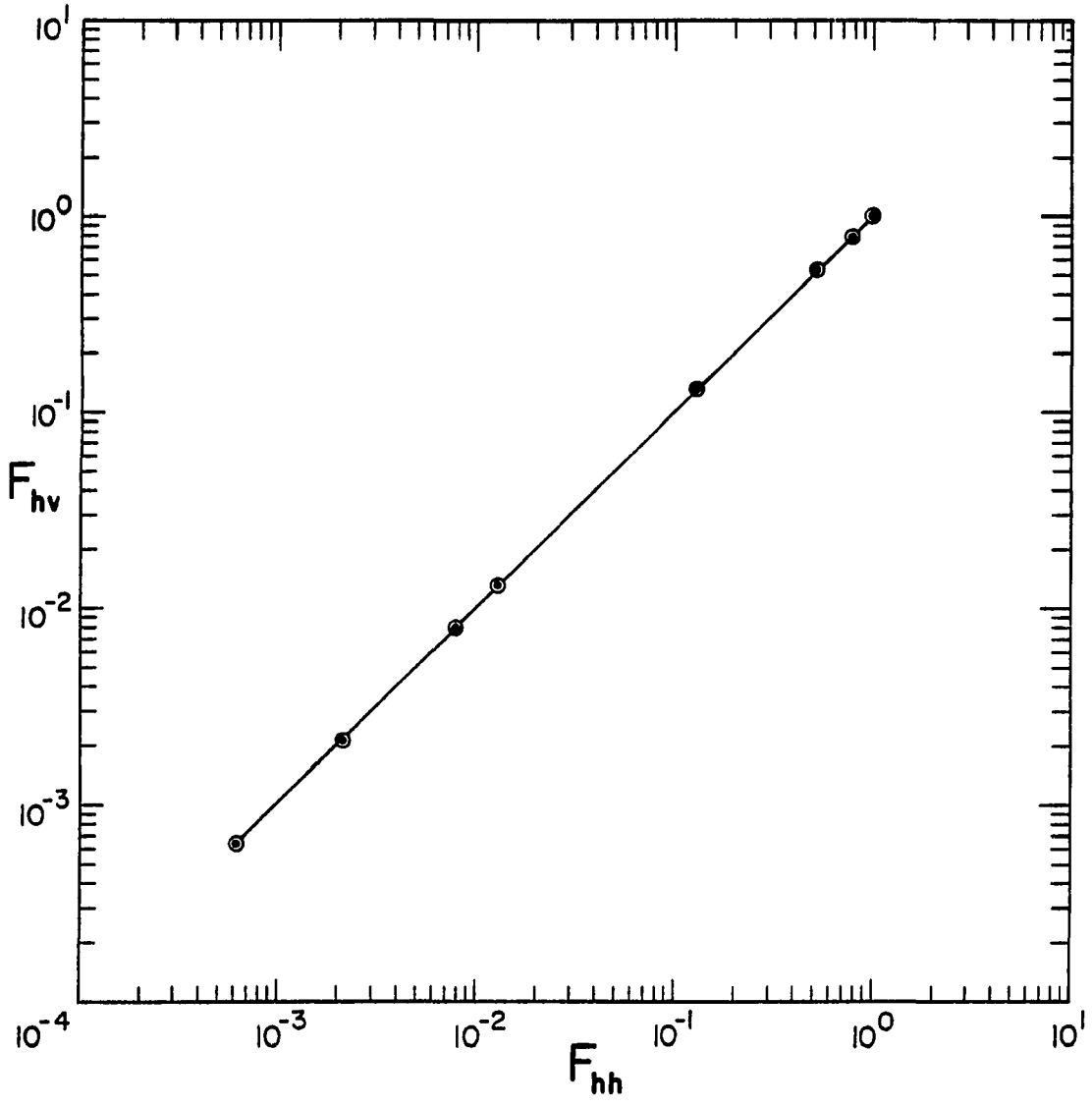


Figure 2.6 Linearity between F_{hv} and F_{hh}. A linearity curve plotted as the log of the vertically versus the log of the horizontally polarized components of fluorescence using a 100 nM solution of rhodamine B in glycerol. Excitation was with horizontally polarized light at 546 nm (band pass, 1.5 nm) and fluorescence detected through a Corning CS 3-66 glass filter. Points were taken by attenuating the incident light. Temperature = 22° C.

was on during the duration of the experiment while the flow was stopped 5 s prior to the actinic flash. The latter was provided by a saturating xenon flash (pulse-width at half-maximum~18 μs) filtered through red glass filters (wavelengths >630 nm). The time constant of the detection system was 10 μs.

2.10 Electron Transport Rates

2.10.1 DCPIP Reduction

The rate of reduction of DCPIP was measured on a Cary 14 recording spectrophotometer provided with a side-illuminator. Actinic light was from a tungsten lamp (Sylvania DVY 650 W, 120 V) through a 5 cm water filter and a glass heat filter; the lamp was operated at 70 V. Excitation in this study was through a 636 nm interference filter (FWHM, 8 nm) and appropriate neutral density filters: the full irradiance at 636 nm was ~10 mW/cm^2. The optical density decrease was measured through an interference filter centered at 597 nm (FWHM, 12.9 nm) with the spectrophotometer set at 597 nm. In the pH studies the extinction coefficients of DCPIP at different pH's were determined as described in Armstrong (1964). Thylakoid suspensions used corresponded to a chlorophyll concentration of ~10 μg/ml; the DCPIP concentration was ~30 μM (cf. Sun and Sauer, 1972).

2.10.2 O_2 Evolution and Uptake

Electron transport rates from water to ferricyanide, dichlorophenolindophenol or oxidized diaminodurene (DAD) were measured as rates of oxygen evolution. Electron transport from ascorbate-reduced DAD

or DCPIP to methylviologen (MV) were measured as rates of oxygen uptake. These O_2 concentration changes with time were measured with a Yellow Springs Instrument platinum/Ag-AgCl Clark type electrode and a Model 53 oxygen monitor. The sample chamber (1.7 ml) was temperature regulated by a surrounding water jacket through which water from a constant-temperature bath was circulated. Excitation was with an incandescent lamp through appropriate filters: full irradiance through a Corning CS 3-70 was 175 mW/cm^2 and that through a 636 nm interference filter (FWHM = 8 nm) was 22 mW/cm^2. All experiments were done at 25 ± 1°C at a chlorophyll concentration of ~25 μg/ml.

Other necessary details of experimental conditions are described in the legends of figures, or at appropriate places in the text.

CHAPTER 3

WAVELENGTH-RESOLVED FLUORESCENCE LIFETIMES AND TRANSIENTS AT 77 K

3.1 Introduction

Plant photosynthesis requires two pigment systems that sensitize two light reactions operating in series [see e.g., Govindjee and Govindjee (1975)]. In order for the overall reaction to operate with the maximum quantum yield, there should be a balanced input of excitation quanta in the two photosystems. It has been proposed that this is somehow regulated by cations [see Barber (1976) and Williams (1977)]. Among the evidence cited for this regulation are the opposite changes in fluorescence intensities from pigment systems I (PS I) and II (PS II), at 77 K, upon cation addition prior to freezing.

In cation-depleted chloroplasts at 77 K, the ratios of intensities of chlorophyll *a* (Chl) fluorescence emitted at 685 nm and 695 nm (F685 and F695) to that at 730 nm (F730) are decreased by the addition of low concentrations (≈10 mM) of NaCl (Gross and Hess, 1973; VanderMeulen and Govindjee, 1974; Wydrzynski *et al.*, 1975), and increased by low concentrations of $MgCl_2$ or high concentrations (≈100 mM) of NaCl (Murata, 1969; Mohanty *et al.*, 1973; Gross and Hess, 1973; VanderMeulen and Govindjee, 1974; Wydrzynski *et al.*, 1975). The above-mentioned changes in fluorescence intensities could be due to changes in Chl *a* fluorescence efficiencies or changes in the populations of the fluorescent species. The former could be the consequence of changes

in the rate constant for excitation transfer from PS II to PS I (Murata, 1969, Butler and Kitajima, 1975a), also known as "spillover" or "redistribution," or in the rate constant of some other radiationless transition (Jennings and Forti, 1974; Malkin and Siderer, 1974). (Here and elsewhere the suffixes II or 2 and I or 1 refer to parameters in PS II and PS I, respectively.) The purpose of the investigation in this chapter is twofold: (a) to test experimentally by lifetime measurements, whether or not the cation-induced changes in relative fluorescence intensities at 77 K are changes in the quantum efficiencies of fluorescence emitted by PS II and/or PS I; and (b) to evaluate, from parallel 77 K fluorescence lifetime and transient measurements, whether or not low concentrations of mono- and di-valent cations could concurrently alter the efficiencies for different pathways of de-excitation of Chl a in the first excited singlet state.

3.2 Materials and Methods

3.2.1 Sample Preparation

Sucrose-washed thylakoid membranes were prepared from chloroplasts of Pisum sativum leaves according to Gross (1971) with minor modifications; 10-minute incubation at 4° C in 100 mM unbuffered sucrose was allowed in each of three wash steps. Concentrated thylakoid suspensions (1-1.5 mg chlorophyll/ml) in 100 mM sucrose were stored in liquid nitrogen until used. Dilutions were made with 100 mM sucrose containing 0.4 mM Tris-HCl at pH 7.6 in all experiments, with the final sample pH in the range of 7.0 ± 0.2. For other details see Chapter 2.

3.2.2 Lifetime Measurements

Fluorescence lifetimes were measured as follows: Fluorescence decays of representative samples were monitored with simple versions of fluorometric systems based on high speed electronic and optoelectronic sampling (Merkelo and Wiczer, 1973; Wiczer and Merkelo, 1975) in order to ascertain that individual fluorescence emission bands decay approximately exponentially. This is an important question to be concerned with in the measurement of fluorescence lifetimes by the phase method. For the present, this does not pose a problem for our measurements as the use of picosecond pulse excitation has shown a nearly exponential decay for PS I fluorescence (Searle *et al.*, 1977) and an exponential decay for PS II fluorescence (F685 and F695) (Campillo and Shapiro, 1978). Upon this confirmation, all lifetime data reported here were obtained by the phase-delay method as described in Chapter 2.

3.2.3 Transient Measurements

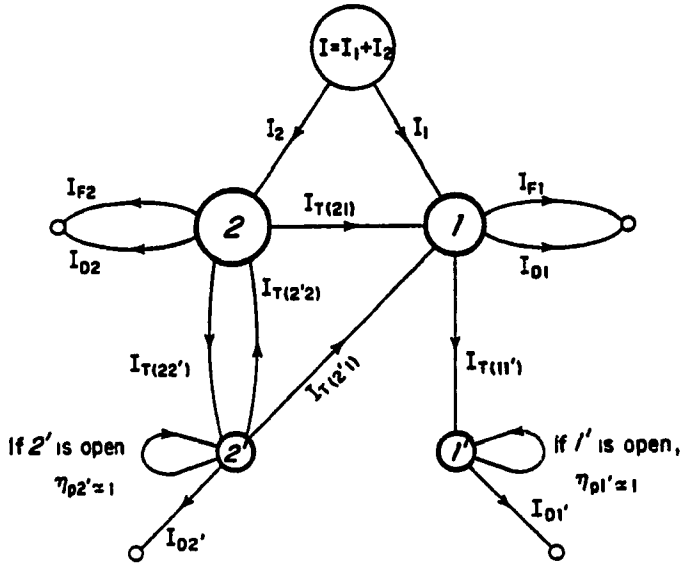
Thylakoid samples were dark adapted for 10 minutes at room temperature and quickly frozen in complete darkness just before a measurement. The experimental details were as given in Chapter 2.

3.2.4 Model for Data Analysis

In thylakoid membranes, chlorophylls exist in one of the five complexes: reaction center I complex, reaction center II complex, Chl *a* antenna complex of PS I (Chl a_I) and of PS II (Chl a_{II}), and the light-harvesting Chl *a*/*b* protein complex

(Chl LH) (see Boardman *et al.*, 1978), the last three serving as antenna for the photosynthetic apparatus. The simplest kinetic formulation for Chl *a* fluorescence at a system level in thylakoids at 77 K is that of a two fluorescent pool or bipartite model, where Chl a_I and part of Chl LH belong to one pool representing the PSI antenna, and Chl a_{II} and the other part of Chl LH belong to the other pool representing the functional PS II antenna (for justification, see Campillo *et al.*, 1977, and Boardman *et al.*, 1978).

Figure 3.1 is a generalized diagram of a bipartite model for energy (or quanta) distribution and redistribution among the two pigment systems in chloroplasts at 77 K. The PS I antenna complex is denoted by $\mathcal{1}$, the set of all its reaction centers by $\mathcal{1}'$, the PS II antenna complex is denoted by $\mathcal{2}$, and the set of all its reaction centers by $\mathcal{2}'$. Capital alphabets in the subscripts denote the pathways--D for radiationless thermal transitions, F for fluorescence, P for photochemistry, and T for energy transfer. For D, F, and P a single numeral is written to the right of the alphabet to define the system--F2 denotes fluorescence from $\mathcal{2}$. In the case of T two numerals are required, the acceptor system being to the right of the donor system--*e.g.*, T(22') denotes excitation transfer from $\mathcal{2}$ to $\mathcal{2}'$ and T(2'2) denotes the back transfer from $\mathcal{2}'$ to $\mathcal{2}$. All pathways for energy dissipation proposed by Butler and Kitajima (1975a,b) are retained in Fig. 3.1; two additional pathways D1' and D2' are added to allow for possible energy dissipation at the closed reaction centers. The empty circles indicate the environment. For processes dependent on the state of the set of PS II reaction centers, $\mathcal{2}'$



$I_2 = \beta I$ $\quad I_1 = \alpha I$

$I_{D2} = I_2\ \eta_{D2}$ $\quad I_{D1} = I_1\ \eta_{D1}$

$I_{F2} = I_2\ \eta_{F2}$ $\quad I_{F1} = I_1\ \eta_{F1}$

$I_{T(21)} = I_2\ \eta_{T(21)}$ $\quad I_{T(11')} = I_1\ \eta_{T(11')}$

$I_{T(22')} = I_2\ \eta_{T(22')}$

$I_{D2'} = I_{T(22')}\ \eta_{D2'}$ $\quad I_{D1'} = I_{T(11')}\ \eta_{D1'}$

$I_{P2'} = I_{T(22')}\ \eta_{P2'}$ $\quad I_{P1'} = I_{T(11')}\ \eta_{P1'}$

$I_{T(2'1)} = I_{T(22')}\ \eta_{T(2'1)}$

$I_{T(2'2)} = I_{T(22')}\ \eta_{T(2'2)}$

Figure 3.1 System-Level Graph of the Photochemical Apparatus. Bold circles denote structural entities and light circles denote the environment. $\mathcal{1}$ denotes the photosystem I antenna complex (Chl a_I + Chl LH), $\mathcal{1}'$ denotes the photosystem I reaction center complex, $\mathcal{2}$ denotes the photosystem II antenna complex (Chl a_{II} + Chl LH), and $\mathcal{2}'$ denotes the photosystem II reaction center complex. All other notations are given according to the convention described in the text and in the Glossary of Terms. (Also see Analysis 1 and 2.)

(intra-$\mathcal{2}'$ interactions unspecified) subscript "(0)" is used when $\mathcal{2}$ is open and "(M)" is used when it is closed (i.e. all reaction centers are as $P680 \cdot Q^-$, where P680 and Q are the primary electron donor and acceptor of photosystem II). The total number of photons absorbed per unit time by the complete photochemical apparatus is denoted by I--I_1 by $\mathcal{1}$, and I_2 by $\mathcal{2}$. Neglecting direct absorptions by $\mathcal{1}'$ and $\mathcal{2}'$, $I = I_1 + I_2$, $I_1 = \alpha I$, $I_2 = \beta I$, and $\alpha + \beta = 1$, where α is the fraction that goes to PS I, and β is the remaining fraction that goes to PS II. With specific reference to the antenna system of PS II, $\mathcal{2}$, the four competing processes in $\mathcal{2}$ are: radiationless transition (k_{D2}), fluorescence (k_{F2}), excitation transfer to $\mathcal{2}'$ ($k_{T(22')}$), and excitation transfer to $\mathcal{1}$ ($k_{T(21)}$). The efficiency (η) for excitation transfer from $\mathcal{2}$ to $\mathcal{1}$ is given by:

$$\eta_{T(21)} = k_{T(21)}/\Sigma k_2 \tag{3.1a}$$

where $\Sigma k_2 = k_{D2} = k_{F2} + k_{T(21)} + k_{T(22')}$. The quantum yield ($\phi$) of excitation transfer from $\mathcal{2}$ to $\mathcal{1}$, defined as the number of quanta transferred from $\mathcal{2}$ to $\mathcal{1}$ divided by the number of quanta absorbed by $\mathcal{2}$, increases from a minimum value of $\phi_{T(21)(0)}$, when the reaction center system $\mathcal{2}'$ is open, to a maximum value of $\phi_{T(21)(M)}$, when $\mathcal{2}'$ is closed. Specifically,

$$\phi_{T(21)(0)} = I_2\eta_{T(21)}/I_2 = \eta_{T(21)} \tag{3.1b}$$

--that is, the efficiency and the quantum yield of excitation transfer from $\mathcal{2}$ to $\mathcal{1}$ are identical when $\mathcal{2}'$ is fully open--and

$$\phi_{T(21)(M)} = \phi_{T(21)(0)} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}] \qquad (3.1c)$$

the enhancement factor in the square brackets originating from the possibility for back transfer of quanta from the closed $\mathcal{Q}'$ to $\mathcal{Q}$ (Butler and Kitajima, 1975a,b). Also, within the framework of the present analysis, the experimentally measured lifetimes of chlorophyll a fluorescence from $\mathcal{Q}$ are given by

$$\tau_{F2(0)} = (\Sigma k_2)^{-1} \qquad (3.2a)$$

and

$$\tau_{F2(M)} = \tau_{F2(0)} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}] \qquad (3.2b)$$

The cause of the lengthening in measured lifetime in Eq. 3.2b is the excitation cycling between $\mathcal{Q}$ and $\mathcal{Q}'$ when the $\mathcal{Q}'$ is closed (i.e. the PS II reaction centers are in the $P680^{+} \cdot Q^{-}$ or $P680 \cdot Q^{-}$ state). Expressing $\tau_{F2(0)}$ and $\eta_{T(22')}$ in terms of rate constants and simplifying, Eq. 3.2b may be rewritten as

$$\tau_{F2(M)} = [k_{D2} + k_{F2} + k_{T(21)} + k_{T(22')}(1 - \eta_{T(2'2)})]^{-1} \qquad (3.2c)$$

This implies that the energy recycling resulting from reaction center closure effectively reduces the rate constant for excitation transfer from the PS II antenna $\mathcal{Q}$ to the reaction center $\mathcal{Q}'$ from $k_{T(22')}$ to $k_{T(22')}(1 - \eta_{T(2'2)})$. It is clear from Eqs. 3.1a and 3.2a that if $\eta_{T(21)}$ and $\tau_{F2(0)}$ are known, the value of $k_{T(21)}$ can be obtained.

In the case of PS I, the lifetime of fluorescence is assumed to be independent of the redox state of the reaction center (Vredenberg and Slooten, 1967), so that

$$\tau_{F1(0)} = \tau_{F1(M)} = (\Sigma k_1)^{-1} \qquad (3.3)$$

where $\Sigma k_1 = k_{D1} + k_{F1} + k_{T(11')}$. The above picture excludes excitation cycling in PS I (see also Campillo et al., 1977).

3.3 Results

3.3.1 Lifetime of Chlorophyll a Fluorescence, at 77 K, in Thylakoids with and without Cations

The results of our Chl a fluorescence lifetime measurements, at 77 K, when all reaction centers are closed are presented in Table 3.1. For sucrose-washed thylakoids suspended in cation-free medium the fluorescence lifetimes (τ) are 0.4 ns for emission at 686 nm, to be referred to as $\tau(F686_{(M)})$, 0.8 ns for $\tau(F695_{(M)})$, and 2.0 ns for $\tau(F730_{(M)})$. Additional measurements at 680 nm ($F680_{(M)}$) give the same results as for $\tau(F686_{(M)})$.

Both F680 and F686 monitor mainly fluorescence associated with Chl a complexes of the light-harvesting Chl a/b complex (Chl LH) and of the other antenna complex of PS II (Wessels et al., 1973; Thornber and Highkin, 1974; Rijgersberg et al., 1978), F695 mainly with the Chl a present only in the antenna complex of PS II (Chl a_{II}), and F730 mainly with the Chl a antenna complex of PS I (Chl a_I) (see Govindjee and Yang, 1966; Govindjee et al., 1967; Butler and Kitajima, 1975a,b;

TABLE 3.1

Effects of Cations on Chlorophyll a Fluorescence Lifetime at 77 K

Sample	Lifetime, τ, nanoseconds*		
	$\tau(F686_{(M)})$**	$\tau(F695_{(M)})$	$\tau(F730_{(M)})$
Salt-depleted	0.42 ± 0.04	0.77 ± 0.04	2.02 ± 0.04
+10 mM NaCl	0.43 ± 0.04	0.77 ± 0.03	2.16 ± 0.08
+10 mM NaCl + 10 mM $MgCl_2$	0.60 ± 0.06	0.99 ± 0.07	2.10 ± 0.07

Washed thylakoids suspended in 100 mM sucrose containing 0.4 mM Tris-HCl at pH 7.6, with a final chlorophyll concentration of about 25 μg/ml and pH 7.0 ± 0.2; samples frozen in a 1 mm cuvette submerged in liquid nitrogen in a Dewar flask; fluorescence measured from front surface.

*The results presented are the mean from five separate measurements from three different batches of chloroplast preparations. The uncertainties in the mean values of τ are standard errors. The experimental precision for each measurement is better than 10 ps, and the same trend for the cation effects existed in every set of samples.

**Fluorescence is detected through interference filters: at 686 nm (FWHM, 6.8 nm) for $\tau(F686_{(M)})$ at 695 nm (FWHM, 6.3) for $\tau(F695_{(M)})$ and at 730 nm (FWHM, 8.4 nm) for $\tau(F730_{(M)})$ in combination with a Schott RG5 cut-off filter (thickness, 3 mm).

Strasser and Butler, 1977a). The addition of 10 mM NaCl (to be referred to as Na^+, henceforth) to salt-depleted thylakoids does not change $\tau(F686_{(M)})$ or $\tau(F695_{(M)})$, but slightly (7% ± 4%) increases $\tau(F730_{(M)})$. Subsequent addition of 10 mM $MgCl_2$ (Mg^{2+}), however, increases τ(F686) by 40 ± 19% and τ(F695) by 29 ± 10% compared to the Na^+ sample, but has no significant effect on $\tau(F730_{(M)})$.

3.3.2 Comparison of Lifetimes of Chlorophyll a Fluorescence, at 77 K, with Fluorescence Intensities in Thylakoids with and without Cations

From the lifetime values in Table 3.1 and the experimental values of maximum (M) fluorescence intensities at 690 and 730 nm, F690 and F730, the lifetime and intensity ratios, $\tau(F686_{(M)})/\tau(F730_{(M)})$, $\tau(F695_{(M)})/\tau(F730_{(M)})$, and $F690_{(M)}/F730_{(M)}$ are calculated and presented in Table 3.2. For comparison, the ratios for the $Na^+ + Mg^{2+}$ samples have also been normalized to one. Two aspects are of interest here. Firstly, the relative lifetime-ratios show that thylakoids depleted of salts or with Na^+ added have the ratios of $\tau(F686_{(M)}$ or $F695_{(M)})/\tau(F730_{(M)})$ 20-30% lower than those suspended in the presence of $Na^+ + Mg^{2+}$. Since salt-depleted and Na^+ samples have lifetimes at 730 nm (mainly from PS I) within only 5% of those in $Na^+ + Mg^{2+}$ samples (Table 3.1 last column), these changes in $\tau(F686_{(M)})/\tau(F730_{(M)})$ and $\tau(F695_{(M)})/\tau(F730_{(M)})$ are accounted for mainly by changes in the lifetimes, and, hence, the efficiencies, of fluorescence from PS II (F686 and F695). Secondly, the relative intensity-ratios for the salt-depleted and Na^+ samples are much lower than the corresponding

TABLE 3.2

Effects of Cations on 77 K Chlorophyll *a* Fluorescence from Photosystem II to Photosystem I Lifetime- and Intensity-Ratios When Reaction Centers of Photosystem II are Closed

	$\tau(F686_{(M)})/\tau(F730_{(M)})$		$\tau(F695_{(M)})/\tau(F730_{(M)})$		$F690_{(M)}/F730_{(M)}$	
Sample	Observed	Normalized	Observed	Normalized	Observed	Normalized
Salt-depleted	0.21	0.72	0.38	0.81	0.31	0.65
+10 mM NaCl	0.20	0.69	0.36	0.76	0.23	0.48
+10 mM NaCl +10 mM $MgCl_2$	0.29	1.00	0.47	1.00	0.48	1.00

lifetime-ratios. For example, the intensity-ratio of the Na^+ samples relative to the $Na^+ + Mg^{2+}$ samples is 0.48, while the relative lifetime-ratios are 0.69 and 0.76 (last two rows in Table 3.2).

3.4 Analysis of Data and Discussion

3.4.1 General Remarks

One objective of the present study was to evaluate, for the first time, from parallel fluorescence lifetime and relative yield measurements, the validity of using the F(680-695)/F730 ratio as an indicator of energy redistribution between the two photosystems. To obtain a comprehensive understanding of the cation-induced changes in fluorescence yields and lifetimes three variations in the extent of electronic interaction in the light-harvesting components of the two photosystems will be considered. With increasing number of interactions the models consist of two fluorescent Chl-protein complexes forming the antenna complexes of PSI and PSII with (A) no electronic energy exchange between the photosystems, (B) energy transfer from the PSII antenna to the PSI antenna, and (C) energy transfer from both the PSII antenna and closed reaction center II to PSI. It will be shown that, irrespective of the extent of interaction, the cation-induced changes in PSII/PSI intensity- and lifetime-ratios must imply some change in the initial sensitization of the two photosystems (i.e., the initial distribution of quanta to PSI and PSII). The second objective of this work was to show that, independent of the ratio changes above, cation-induced changes in the lifetimes of fluorescence imply changes in the rate constant(s) of

non-radiative transition(s), including excitation energy transfer from PSII to PSI.

3.4.2 Analysis of Parallel Fluorescence Lifetime and Transient Results

To facilitate discussion, each of the above cases will be considered as a special case of the general scheme for the photosynthetic apparatus in Fig. 3.1. (Note that the assignment of Chl LH to the antenna of both PSI and PSII is a departure from the earlier models of Butler and coworkers (Butler and Kitajima, 1975a,b; Butler and Strasser, 1977a).)

CASE A: NO EXCITATION ENERGY TRANSFER BETWEEN THE TWO PHOTOSYSTEMS. This implies that both $I_{T(21)}$ and $I_{T(2'1)} = 0$ in Fig. 3.1. The maximum yield of fluorescence from the antenna complex of PSII (2) (see Materials and Methods, Eq. 3.2c) is given by $F_{2(M)} = \beta I\, k_{F2}/[k_{D2} + k_{T(21')}(1 - \eta_{T(2'2)})] = \beta I k_{F2} \tau_{F2(M)}$, where all the terms have the meaning defined under Materials and Methods in this chapter and in Glossary of Terms. Similarly, for PSI, $F_{1(M)} = \alpha I k_{F1} \tau_{F1(M)}$. Since the rate constant for fluorescence (k_F) is a molecular property unlikely to be affected by the small perturbations of suspension medium in this study, it is taken that $k_{F1} = k_{F2} = k_F$ (For justification that k_F remains constant, see Footnote*). Then, with the aid of the relation

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*Constancy of k_F had also been assumed in the study of Chl *a* fluorescence in the past (Murata, 1969; Briantais *et al.*, 1973; Malkin and Siderer, 1974) because of constancy of absorption and fluorescence spectra of the samples. In view of reports (Gross and Hess, 1973; and Henkin and Sauer, 1977) that the absorption spectra vary in different cationic conditions, we measured the absorption spectra in our samples. Our results (Fig. 2.1) reveal that the changes in absorption spectra

$\alpha + \beta = 1$, the fluorescence intensity-ratio is given by

$$\frac{F_{2(M)}}{F_{1(M)}} = \frac{\beta}{1 - \beta} \cdot \frac{\tau_{F2(M)}}{\tau_{F1(M)}} \qquad (3.4)$$

Changes in β by cation addition explains the discrepancies between $F_{2(M)}/F_{1(M)}$ and $\tau_{F2(M)}/\tau_{F1(M)}$ (Table 3.2). A comparison of $F690_{(M)}/F730_{(M)}$ and $\tau(F686_{(M)})/\tau(F730_{(M)})$ give β = 0.60, 0.53, and 0.62 for the salt-depleted, Na^+, and $Na^+ + Mg^{2+}$ samples, respectively; the corresponding values for β using the $\tau(F695_{(M)})/\tau(F730_{(M)})$ values in Table 3.2 are 0.45, 0.39, and 0.51. The differences in the calculated absolute values of β are the consequence of the simplicity of the model. The major point, however, is clear; the fluorescence intensity- and lifetime-ratio discrepancies can readily be accounted for if the relative sensitization of PSII fluorescence decreases by ~10% with Na^+ addition and increases by 15-30% with Mg^{2+} addition. Such changes in β are in excellent agreement with our conclusions from cation effects on the saturation of delayed light emission (Wong *et al.*, 1978--Chapter 4). A partial cause of this change has now been shown to be the greater extent of excitation transfer from Chl *a* and Chl *b* (in Chl LH) to Chl a_{II} (Wong and Govindjee, 1978--Chapter 5). However, to explain the changes in the absolute lifetime values additional changes must occur (see below).

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are small, and that most of these changes (decreases at the absorption peaks and increases at the "valleys") appear to be the consequence of the "sieve effect" (Duysens, 1956). Thus, to a first approximation, the assumption of the constancy of k_F appears sound.

In the experiments reported in Table 3.1 the incident irradiance was sufficient to close all the reaction centers, and the lifetimes of $F686_{(M)}$ and $F695_{(M)}$ are given by Eq. 3.2c and that of F730 by Eq. 3.3. These lifetime equations may be rewritten as

$$\tau_{Fi(M)} = (k_{Di} + k_{Fi} + k'_{T(ii')})^{-1} \quad i = 1 \text{ or } 2 \tag{3.5}$$

where the rate constants k_{Di} and k_{Fi} are as previously defined, and $k'_{T(ii')}$ is the <u>effective</u> rate constant for excitation transfer from the antenna to the trap, with $k'_{T(11')} = k_{T(11')}$ and $k'_{T(22')} = k_{T(22')}(1 - \eta_{T(2'2)})$. The addition of Na^+ to salt-depleted thylakoids results in no change in $\tau(F686_{(M)})$ or $\tau(F695_{(M)})$ (Table 3.1). Within the context of the present model this implies either that the rate constants are unaffected by low concentrations of Na^+ or that changes in k_{D2} and $k'_{T(22')}$ fortuitously compensate each other; k_{F2} is constant as before. Subsequent addition of Mg^{2+} induces an increase in both $\tau(F686_{(M)})$ and $\tau(F695_{(M)})$ (last row in Table 3.1) implying a decrease in $(k_{D2} + k'_{T(22')})$. The latter is consistent with out previous conclusion (Wong <u>et al.</u>, 1978--Chapter 4) from fast fluorescence transients that the addition of Mg^{2+} reduces the rate constants for radiationless processes in the antenna chlorophylls in $\mathcal{Q}$ (radiationless de-excitation, k_{D2}, or transfer to the reaction center, $k_{T(22')}$) and/or radiationless dissipation at a closed trap--affecting, in this analysis, the term $(1 - \eta_{T(2'2)})$. In the case of F730, Na^+ addition leads to a slight increase in $\tau(F730_{(M)})$ implying that $(k_{D1} + k_{T(11')})$ is decrease by 7 ± 4%. The relatively insignificant effect upon subsequent addition of Mg^{2+}

suggests that $(k_{D1} + k_{T(11')})$ then remains constant.

CASE B. EXCITATION TRANSFER FROM PSII TO PSI OCCURS THROUGH THE ANTENNA COMPLEXES (SINGLE-SPILLOVER MODEL). This case implies that $I_{T(2'1)} = 0$ in Fig. 3.1. The first step in this analysis is to estimate the fraction of quanta absorption by PSI, α. In order to achieve this, using 77 K fluorescence transient and lifetime results, we have derived a new formulation for α (details given in Analysis 1). The use of this new equation

$$\alpha = \frac{F_{1(\alpha)}}{F_{1(\alpha)} + \frac{\tau_{F1(M)}}{\tau_{F2(M)}} \cdot F_{2(M)}} \tag{3.6}$$

represents the main departure from the original calculations of Butler and Kitajima (1975a,b) and Butler (1977). (The identity between our Eq. 3.6 and the alternate, Eq. 9 of Strasser and Butler (1977a), to calculate α is shown in Analysis 2.) Table 3.3 summarizes the results of this analysis; the main findings are: (a) a Mg^{2+}-induced decrease in the efficiency of energy transfer from PSII to PSI $(\eta_{T(21)})$--~50% change; (b) a Mg^{2+}-induced increase in the sum of efficiencies of non-radiative de-excitation in the PS II antenna and of energy transfer from this antenna to the reaction center $(\eta_{D2} + \eta_{T(22')})$--~20% change, and (c) an effect of cations on the initial distribution of quanta to the photosystems--~6% decrease in β by Na^+, and ~12% subsequent increase by Mg^{2+}. The last column in Table 3.3 shows that the expected values of $F690_{(M)}/F730_{(M)}$ correspond closely to the measured ratios in Table 3.2 demonstrating that the energy distribution and

TABLE 3.3

Effects of Cations on the Excitation Distribution and Redistribution Parameters in Salt-Depleted Thylakoids by the Single-Spillover Model (See Glossary of Terms)

Sample	α	β	η_{F2}	$\eta_{T(21)}$	$\eta_{D2} + \eta_{T(22')}$	Calculated $F690_{(M)}/F730_{(M)}$
Salt-depleted	0.36	0.64	0.02	0.25	0.73	0.29
+10 mM NaCl	0.40	0.60	0.02	0.27	0.71	0.24
+10 mM NaCl +10 mM $MgCl_2$	0.33	0.67	0.02	0.13	0.85	0.48

In the table, α is calculated by Eq. 3.6 and $\beta = 1 - \alpha$. $\eta_{F2} = \tau_{F2(0)}/\tau_o$, where $\tau_{F2(0)} = \tau_{F2(M)} \cdot F690_{(0)}/F690_{(M)}$, and τ_o (the lifetime of fluorescence in the absence of competition is taken to be 15.2 ns (Brody and Rabinowitch, 1957); $\eta_{T(21)}$ is calculated by Eq. 18A, and $(\eta_{D2} + \eta_{T(22')})$ is obtained using the condition $\eta_{D2} + \eta_{F2} + \eta_{T(21)} + \eta_{T(22')} = 1$. $F690_{(M)}/F730_{(M)}$ is calculated as:

$$[\beta \cdot \tau(F690_{(M)})/\tau(F730_{(M)})]/[\alpha + \beta \cdot \eta_{T(21)} \cdot (1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}].$$

redistribution parameters in columns 2 to 4 in Table 3.3 are consistent with the empirical observations.

Within the sensitivity of our measurements, the efficiency for fluorescence from $\mathcal{L}$, when $\mathcal{L}'$ is open, is unaffected by 10 mM mono- and di-valent cations. This finding results from the following considerations. The values in Table 3.1 correspond to $\tau_{F2(M)}$ (see Eq. 3.2b), and $\tau_{F2(0)}$ can be calculated from Eqs. 3.2a,b, and 20A as $\tau_{F2(M)} \cdot F690_{(0)}/F690_{(M)}$ (Analysis 1). If it is assumed that $\tau_{F2(M)} = \tau(F690_{(M)}) = \frac{1}{2}[\tau(F685_{(M)}) + \tau(F695_{(M)})]$, then $\tau_{F2(M)} = 0.6$, 0.6, and 0.8 ns and $\tau_{F2(0)} = 0.35$, 0.34, 0.35 ns for the salt-depleted, Na^+, and $Na^+ + Mg^{2+}$ samples. Since by Eq. 3.2a, $\tau_{F2(0)} = (\Sigma k_2)^{-1}$, the above results show that Σk_2 is invariant under the different cationic conditions. By taking the intrinsic lifetime of Chl a fluorescence in the absence of competing processes ($\tau_o = k_{F2}^{-1}$) to be 15.2 ns (Brody and Rabinowtich, 1957), the efficiency of fluorescence is given by $\tau_{F2(0)}/\tau_o$ as $\simeq 0.02$. It must be stated that the use of Eqs. 3.2b and 20A, which assume the concept of excitation cycling between $\mathcal{L}$ and $\mathcal{L}'$, leads to the result that Σk_2 is invariant.

CASE C. ENERGY REDISTRIBUTION OCCURS FROM THE ANTENNA AND CLOSED REACTION CENTER OF PSII TO PSI (DUAL-SPILLOVER MODEL). This is the case shown in Fig. 3.1. The analysis of this model was developed by Butler and Kitajima (1975a) and Butler (1977) and are included, in the context of our model, in Analysis 2. As in case B, our new approach is in the calculation of α using Eq. 3.6. The kinetic analysis by this model contains one more parameter than available and, thus, an

assumption must be made for the value of the efficiency of energy transfer from the PS II antenna complex $\mathcal{Q}$ to the reaction center $\mathcal{Q}'$, $\eta_{T(22')}$. For any one sample, if the empirically obtained value for $\eta_{T(22')}\ \eta_{T(2'2)}$, from $F_{2(v)}/F_{2(M)}$ (Eq. 19A in Analysis 1, is x, the lower limit of $\eta_{T(22')}$ is x, when $\eta_{T(2'2)}$ attains its maximum value of 1. The upper limit for $\eta_{T(22')}$ is determined by the boundary condition for $\mathcal{Q}$, that $\eta_{D2} + \eta_{F2} + \eta_{T(21)} + \eta_{T(22')} = 1$. In instances where more than a single sample is of interest, additional information on the relative values of $\eta_{T(22')}$ of the different samples must be known. In the absence of this information two extreme cases can be considered (see discussion below): at one limit, $\eta_{T(22')}$ is independent of the cationic content of the suspension medium, being a function only of the thylakoid preparation (case C.1); at the other limit, $\eta_{T(22')}$ is also a function of the cationic composition of the suspension medium (case C.2).

3.4.3 Cation Effects on Excitation Distribution and Redistribution between the Two Pigment Systems

First, we need to establish the interrelations among β, η_{D2}, $\eta_{T(21)}$, and $\eta_{T(22')}$. The functional dependence of η_{D2} on $\eta_{T(22')}$, $\eta_{T(21)}$ on $\eta_{T(22')}$, and η_{D2} on $\eta_{T(21)}$ over the complete range of admissible values of $\eta_{T(22')}$ are presented in Figs. 3.2, 3.3, and 3.4, respectively. It is seen in Fig. 3.2, that for the salt-depleted and Na^+ samples, η_{D2} increases slightly with increasing $\eta_{T(22')}$ at the lower range of admissible values of $\eta_{T(22')}$ (0.41-0.45 in salt-depleted

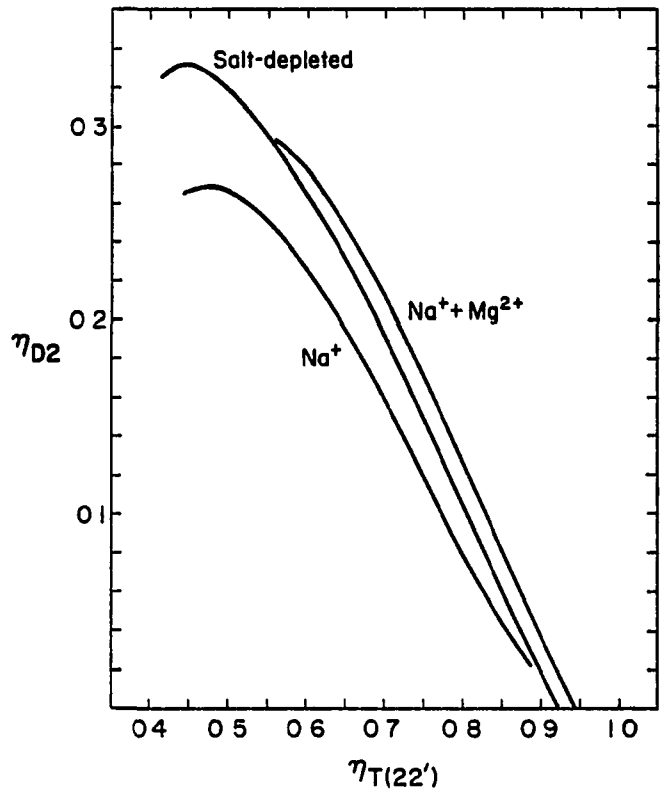

Figure 3.2 Relation between η_{D2} and $\eta_{T(22')}$. The efficiency of thermal loss in the antenna complex of PS II (η_{D2}) as function of the efficiency of excitation energy transfer from the antenna complex of PS II to its reaction center complex ($\eta_{T(22')}$) over the range of all admissible values of $\eta_{T(22')}$ for sucrose-washed thylakoids under different cationic conditions. The suspension medium for the "salt-depleted" samples (middle curve) consists of 100 mM sucrose + 0.4 mM Tris-HCl, pH 7.6, and the "Na^+" and "$Na^+ + Mg^{2+}$" samples contain in addition 10 mM NaCl (lower curve) and 10 mM NaCl + 10 mM $MgCl_2$ (upper curve), respectively. The determination of the range of admissible values of $\eta_{T(22')}$, for any sample, from the empirical results is outlined in the text.

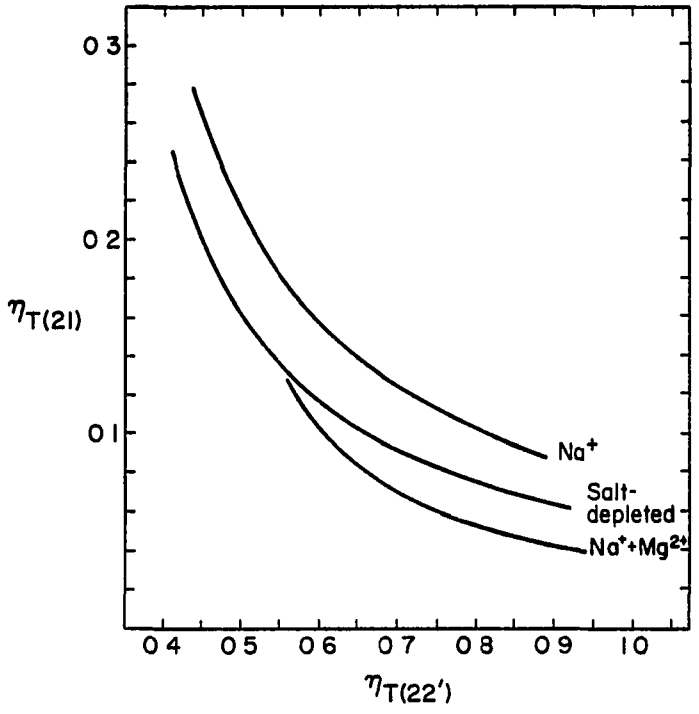

Figure 3.3 Relation between $\eta_{T(21)}$ and $\eta_{T(22')}$. The efficiency of excitation energy transfer from the PS II antenna complex to the PS I antenna complex ($\eta_{T(21)}$) as function of the efficiency of excitation energy transfer from the antenna complex of PS II to its reaction center complex ($\eta_{T(22')}$) for all admissible values of the latter. Other details are given in the legend of Fig. 3.2.

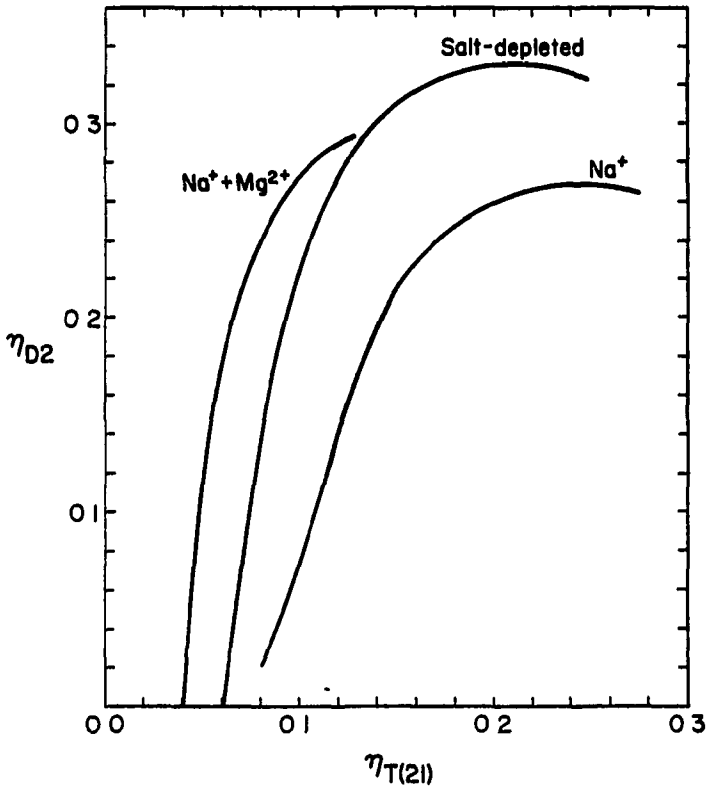

Figure 3.4 Relation between η_{D2} and $\eta_{T(21)}$. The efficiency of thermal loss in the antenna complex of PS II (η_{D2}) as function of the efficiency of excitation energy transfer from the PS II antenna complex to the PS I antenna complex ($\eta_{T(21)}$) over their allowed values. Other details are given in the legend of Fig. 3.2.

samples and 0.44-0.48 in Na^+ samples), and that η_{D2} decreases for all other admissible values of $\eta_{T(22')}$. In Fig. 3.3, $\eta_{T(21)}$ also decreases with increasing $\eta_{T(22')}$ but with decreasing slope (concave curves). The plot of η_{D2} versus $\eta_{T(21)}$ in Fig. 3.4 reveals that in going from the lower to the upper limit of $\eta_{T(21)}$, η_{D2} first increases more rapidly than $\eta_{T(21)}$ (slope > 1) and then levels off, with $\eta_{D2} > \eta_{T(21)}$ in almost all instances. Unlike the η's, the fraction of quanta distributed to $\mathcal{L}$ (β) shows no dependence on the value of $\eta_{T(22')}$--for example, in Table 3.4 (column 3) it is seen that β varies from 0.67 to 0.64 (a change of <4%, within the limits of uncertainty in this study) over the range of allowed values of $\eta_{T(22')}$ (0.56 to 0.94) for the $Na^+ + Mg^{2+}$ samples.

THE CASE C.1. Since a way of uniquely determining the value of $\eta_{T(22')}$ is not available at this time, the two cases for the cationic dependence of $\eta_{T(22')}$ mentioned above will be considered separately. In the first case in which $\eta_{T(22')}$ is a fixed property of any one thylakoid preparation and is independent of the cationic composition of the suspension medium, the effects of cations on η_{D2} and $\eta_{T(21)}$ can readily be obtained from Figs. 3.2 and 3.3. For any value of $\eta_{T(22')}$, the intersection of the vertical line corresponding to the value of $\eta_{T(22')}$ and the curves in the η_{D2} versus $\eta_{T(22')}$ and $\eta_{T(21)}$ versus $\eta_{T(22')}$ plots give the values for η_{D2} and $\eta_{T(21)}$ for the different samples. For example, if $\eta_{T(22')} = 0.7$, Fig. 3.2 gives η_{D2} (salt-depleted) = 0.19; the addition of 10 mM Na^+ decreases η_{D2} to 0.16 and subsequent addition of 10 mM Mg^{2+} increases η_{D2} to 0.21. On the other

hand, when $\eta_{T(22')} = 0.7$, $\eta_{T(21)}$ (salt-depleted) = 0.09; addition of 10 mM Na^+ increases $\eta_{T(21)}$ to 0.12 and subsequent addition of 10 mM Mg^{2+} decreases $\eta_{T(21)}$ to 0.07. The corresponding $k_{T(21)}$ values, using Eqs. 3.1a and 3.2a, are 2.6 x $10^8 s^{-1}$, 3.5 x $10^8 s^{-1}$ and 2.0 x $10^8 s^{-1}$, respectively. It is clear from Figs. 3.2 and 3.3 that similar results are expected for all allowed values of $\eta_{T(22')}$ in this case. Furthermore, these values of $k_{T(21)}$ are appropriate for an inductive resonance mechanism for energy transfer (cf. Förster, 1965; Shipman and Housman, 1979).

THE CASE C.2. The analysis in the second case, when $\eta_{T(22')}$ is also a function of the cationic content of the suspension medium, is a little less direct in that the relative values of $\eta_{T(22')}$ for the different samples to be compared must be known. If it is taken that the relative values of $\eta_{T(22')}$, given by the ratio $\eta_{T(22')}$ (sample)/$\eta_{T(22')}$ ($Na^+ + Mg^{2+}$), are 0.88, 0.83, and 1.00 for the salt-depleted, 10 mM NaCl, and 10 mM NaCl + 10 mM $MgCl_2$ samples, respectively--based on the fraction of PS II reaction centers undergoing charge separation in a non-saturating flash (cf. Wong et al., 1978; Chapter 4)--the results in Table 3.4 are obtained. It should be noted that, unlike the previous case, to obtain η_{D2} and $\eta_{T(21)}$ for the samples in Figs. 3.2 and 3.3, three different values for $\eta_{T(22')}$ must be used, one for each cationic condition. The main findings from this analysis, given in Tables 3.4 and 3.5, are the following:

(1) for all allowed values of $\eta_{T(22')}$($Na^+ + Mg^{2+}$), the addition of 10 mM Na^+ to salt-depleted thylakoids causes a relatively insignificant

TABLE 3.4

Effects of Cations on the Excitation Distribution and Redistribution Parameters in Salt-Depleted Thylakoids for Different Values of $\eta_{T(22')}(Na^+ + Mg^{2+})$ by the Dual-Spillover Model (See Glossary of Terms)

		1	2					2'
	Sample	α	β	η_{D2}	η_{F2}	$\eta_{T(21)}$	$\eta_{T(22')}$	$\eta_{T(2'2)}$
A.	Salt-depleted	0 39	0 61	0.32	0.02	0 17	0.49	0 84
	+ 10 mM NaCl	0.41	0.59	0.27	0 02	0 25	0 46	0.96
	+ 10 mM NaCl + 10 mM $MgCl_2$	0 33	0 67	0.29	0 02	0 13	[0.56]	1.00
B.	Salt-depleted	0 41	0 59	0 19	0 02	0.09	0.70	0 59
	+ 10 mM NaCl	0 45	0 55	0 18	0 02	0 14	0 66	0 67
	+ 10 mM NaCl + 10 mM $MgCl_2$	0.36	0 64	0.13	0 02	0.05	[0 80]	0 70
C.	Salt-depleted	0 42	0 58	0 08	0 02	0 07	0 83	0.51
	+ 10 mM NaCl	0 46	0 54	0 09	0 02	0 11	0 78	0 59
	+ 10 mM NaCl + 10 mM $MgCl_2$	0 37	0.63	0 00	0 02	0 04	[0 94]	0 62

In A, it is assumed that $\eta_{T(22')}(Na^+ + Mg^{2+})$ is 0.56, in B it is 0.80 and in C, it is 0.94. In this table, $\eta_{F2} = \tau_{F2(0)}/\tau_o$, where $\tau_{F2(0)} = \tau_{F2(M)}[F_{2(0)}/F_{2(M)}]$ and τ_o (the intrinsic lifetime of chlorophyll a fluorescence) is assumed to be 15.2 ns (Brody & Rabinowitch, 1957); $\eta_{T(21)}$ is given by Eq. (18A); $\eta_{T(22')}$ (salt-depleted) = 0.88 $\eta_{T(22')}(Na^+ + Mg^{2+})$; $\eta_{T(22')}(Na^+) = 0.83\ \eta_{T(22')}(Na^+ + Mg^{2+})$ for the three values of $\eta_{T(22')}(Na^+ + Mg^{2+})$ given in the boxes; and η_{D2} is obtained from the boundary condition for system 2, $\eta_{D2} + \eta_{F2} + \eta_{T(21)} + \eta_{T(22')} = 1$.

TABLE 3.5

Effects of Cations on the Parameters Affecting Excitation Energy Transfer and Degradation in and between the Systems $\mathcal{1}$, $\mathcal{2}$, and $\mathcal{2}'$ (see Fig. 3.1) (See Glossary of Terms)

	a $[\eta_{D1} + \eta_{T(11')}]$	b $\eta_{T(21)}$	c $[\eta_{D2} + \eta_{T(22')}]$	d $[1-\eta_{T(22')}\eta_{T(2'2)}]^{-1}$	e $\tau_{F2(0)}[1-\eta_{T(22')}\eta_{T(2'2)}]^{-1}$
	radiationless transitions, PS I	excitation transfer PS II PS I	radiationless transitions PS II	maximum excitation cycling enhancement of fluorescence, PS II	maximum fluorescence lifetime, PS II
+ 10 mM NaCl/ salt-depleted	+ 7 ± 4%	+ 47-57%	+ 4-10%	+ 5 ± 3%	No effect
+ 10 mM NaCl + 10 mM $MgCl_2$/ + 10 mM NaCl	No effect	+ 48-64%	+ 5-16%	+ 31 ± 5%	+ 32 ± 16%

[a] $\eta_{D1} + \eta_{T(11')}$ (the sum of efficiencies for all radiationless transitions in the antenna complex of PS I) is determined from the measured chlorophyll *a* fluorescence lifetimes at 730 nm (Table 3.1), the assumption that $k_F^{-1} = 15.2$ ns (Brody and Rabinowitch, 1957), and Eq. 3.3. The uncertainties denote ± one standard deviation for measurements on 5 different sets of samples from 3 batches of chloroplasts.

[b] The effects of cations on $\eta_{T(21)}$ (the efficiency for excitation energy transfer from the antenna complex of PS II to that of PS I, $\mathcal{2} \rightarrow \mathcal{1}$) is determined from column 5 in Table 3.4 and Fig. 3.3. The range of values presented represent the set of values taken by $\eta_{T(21)}$ over the set of allowed values of $\eta_{T(22')}$ $\eta_{T(2'2)}$ ($Na^+ + Mg^{2+}$).

[c] The efficiency of excitation energy transfer from the antenna complex of PS II to its associated reaction center complex, $\eta_{T(22')}$, is unknown, but influences the effects of cations on η_{D2} (the efficiency for thermal loss in the antenna complex of PS II) (see for

example, column 2 in sets A and B in Table 3.4). The sum $[\eta_{D2} + \eta_{T(22')}]$, however, shows a consistent trend of effects by 10 mM mono- and di-valent cations. The ranges of values presented are obtained, as described in b, using Table 3.4 and Fig. 3.2.

[d]This is the excitation "enhancement factor" which reflects the extent of excitation energy cycling between the antenna and reaction center complexes in PS II. The effects of cations on $(1 - \eta_{T(22')} \cdot \eta_{T(2'2)})^{-1}$ is determined from the empirical values for $F690_{(M)}/F690_{(O)}$, taking $F690_{(O)} = F_{2(O)}$ and $F690_{(M)} = F_{2(M)}$ in Eq. 20A.

[e]The term $\tau_{F2(O)}[1 - \eta_{T(22')} \eta_{T(22')}]^{-1}$ based on Eq. 3.2b is calculated from the ratio of the maximum (reaction center II closed) lifetimes at 690 nm, τ(F690). The latter is assumed to be equal to $\frac{1}{2}[\tau(F685) + \tau(F695)]$ (see Table 3.1), and gives 0.6, 0.6, and 0.8 ns for the salt-depleted, Na^+, and $Na^+ + Mg^{2+}$ samples.

↑ denotes increase and ↓ denotes decrease.

Other details of samples are given in the legends of Table 3.1.

(3 to 7%) decrease in β (column 3, Table 3.4) and a large (47 to 57%) increase in $\eta_{T(21)}$ (column 6, Table 3.4; column b, Table 3.5). Subsequent addition of 10 mM Mg^{2+} causes a 13 to 17% increase in β and 48 to 64% decrease in $\eta_{T(21)}$. The most striking difference between these results and those in case B (Table 3.3) is in the effect of Na^+ on $\eta_{T(21)}$--that is, while the analysis by the single-spillover model shows no significant effect of Na^+ on $\eta_{T(21)}$, the analysis by the dual-spillover model shows a large increase.

(2) Concomitant with the above changes, the sum of efficiencies of nonradiative processes in $\mathcal{L}$, taken as $(\eta_{D2} + \eta_{T(22')})$, decreases by 4-10% with the addition of Na^+ and increases by 5-10% upon subsequent addition of Mg^{2+}.

(3) A distinction between the two components η_{D1} and $\eta_{(T11')}$ in the radiationless processes in $\mathcal{L}$ is not possible by this analysis, and the same conclusions reached in case A hold; that is, the addition of Na^+ decreases $(\eta_{D1} + \eta_{T(11')})$ by 7 ± 4%, after which the addition of Mg^{2+} seems to have no further effect.

3.4.4 Excitation Energy Transfer and FLuorescence Lifetime

The formalism for excitation cycling between the antenna complex $\mathcal{L}$ and the reaction center complex $\mathcal{L}'$ on the fluorescence lifetime is, in principle, analogous to the special case of self-absorption of fluorescence with a constant probability of absorption of an emitted photon (cf. Birks and Munro, 1967). The lifetime of the fluorescence from $\mathcal{L}$ when $\mathcal{L}'$ is closed, and excitation cycling is maximum, is longer than

the lifetime in the absence of cycling by the factor $[1 - \eta_{T(22')} \eta_{T(2'2)}]^{-1}$. At the same time, the fluorescence intensity from $\mathcal{Q}$ when $\mathcal{Q}'$ is closed exceeds the intensity when $\mathcal{Q}'$ is open by the same factor $[1 - \eta_{T(22')} \eta_{T(2'2)}]^{-1}$. The cation-induced changes in $[1 - \eta_{T(22')} \eta_{T(2'2)}]^{-1}$ from fluorescence transients and $\tau_{F2(0)} [1 - \eta_{T(22')} \eta_{T(2'2)}]^{-1}$ from lifetime measurements are given in Table 3.5. Both sets of results show that Na^+ has no significant effects on $[1 - \eta_{T(22')} \eta_{T(2'2)}]^{-1}$ but Mg^{2+} addition increases it by ~30%. The one-to-one correspondence of the cation effects in these results again show that $\tau_{F2(0)}$ is constant.

A constant $\tau_{F(0)}$ implies, as stated earlier, that Σk_2, the sum of rate constants of the four competing processes in $\mathcal{Q}$, is constant and that the relative changes in the efficiency of any process in $\mathcal{Q}$ give directly the relative changes in the rate constant for that process. The insensitivity of $\tau(F730_{(M)})$ to $k_{T(21)}$ suggests that the times for energy transfer from Chl $\underline{a}_{II}$ and Chl LH to Chl $\underline{a}_{I}$ are much shorter than $\tau(F730_{(M)})$; this is consistent with the data of Campillo et al. (1977) which show a transfer time of 140 ± 40 ps from Chl LH to Chl $\underline{a}_{I}$, based on the rise-time of F730 in a single picosecond flash excitation. Therefore, depending upon the relative values of $k_{T(21)}$, the intensity of fluorescence at 730 nm could change substantially without any effect on its lifetime. This greater susceptibility of F730 than $\tau(F730)$ to changes in $k_{T(21)}$ readily explains why the fluorescence intensity-ratio is more sensitive than the lifetime-ratio to the cationic conditions of the suspension medium. We recall (from case A) that the discrepancies

in the fluorescence intensity- and lifetime-ratios can also be understood without admitting energy transfer from PS II to PS I. In this case when inter-photosystem energy transfer is excluded, the discrepancies in the ratios are explained by changes in the relative sensitization of the two photosystems. When inter-photosystem excitation transfers are allowed, part of the change attributed to sensitization in the previous model is explained as energy transfer from PS II to PS I.

The results and arguments in favor of redistribution of electronic excitation from PS II to PS I are discussed in detail by Williams (1977). We also favor the existence of cation regulation of excitation redistribution in these samples for the following reason: The idea that low concentrations (10 mM) of Na^+ increase the spillover of energy from $\mathcal{L}$ to $\mathcal{I}$ is supported by the data of Wong and Govindjee (1978--Chapter 5) which show that the degree of polarization of Chl a fluorescence at 23° C at 686 and 730 nm (mainly PS II) and at 712 nm (PS II and PS I) increases and decreases, respectively, upon Na^+ addition. Decrease in spillover by Mg^{2+} is supported by the reversal of the above effects on polarization by subsequent addition of 10 mM Mg^{2+}. Thus, cases B and C (discussed above) are more accurate representations of the light-harvesting process in the photosynthetic apparatus.

The lifetime values presented here for thylakoid membranes from pea chloroplasts containing both Na^+ and Mg^{2+} are 0.6, 1.0, and 2.1 ns for τ(F686), τ(F695), and τ(F730), respectively. They are in fair agreement with ~0.9 and 1.5 ns for τ(F690) and τ(F735) in spinach

thylakoids measured by Campillo et al. (1977) with single picoseond pulses (2×10^{14} photons cm^{-2} $pulse^{-1}$). The τ(F685), measured by Yu et al. (1977), with a train of pulses (2×10^{14} photons cm^{-2} pulse $pulse^{-1}$), are lower, probably because of the presence of long-lived quenchers produced by the mode of excitation. Variations in lifetimes measured at 77 K exist; for example, in thylakoids under different salt conditions, τ(F686) ranges from 0.4 to 0.6 ns and τ(F730) ranges from 2.0 to 2.2 ns, while in different algae (Chlorella, Porphyridium, and Anacystis, see Mar et al., 1972) τ(F685) ranges from 0.7 to 1.4 ns and τ(F730) ranges from 0.8 to 2.3 ns. Thus, differences in lifetimes observed in various laboratories need not be ascribed only to differences in intensities and modes of excitation, but may also result from variations in the physical configurations of the membrane.

3.5 Concluding Remarks

Maximum quantum yield of green plant photosynthesis, under light-limiting conditions, requires the balanced input of electronic excitation into the two photosystems. In thylakoid membranes, the latter, monitored mainly by changes in chlorophyll a fluorescence intensities (F) from the two photosystems, has been suggested to be regulated by cations. Presented here are the first measurements of the cation effects on the lifetime (τ) of chlorophyll a fluorescence at 77 K. In sucrose-washed thylakoid membranes from pea chloroplasts, at neutral pH, τ(F686) (τ at 686 nm), τ(F695), and τ(F730) are 0.4, 0.8, and 2.0 ns, respectively. Addition of 10 mM NaCl increases τ(F730) by ~7% without

any measureable effect on τ(F686) and τ(F695). Subsequent addition of 10 mM $MgCl_2$ increases τ(F686) to 0.6 ns and τ(F695) to 1.0 ns, with no significant effect on τ(F730). In parallel measurements at 77 K, the cation-induced changes in the ratio of relative fluorescence at 690 nm (mainly photosystem II, PS II) to that at 730 nm (mainly PS I) are found to exceed the changes for τ(F686 or F695)/τ(F730). A new method of calculating the fraction of quanta absorbed by PS I (α) using parallel τ and F measurements in a single sample is presented. Analyses of results show that cations regulate concomitant changes in (a) excitation energy transfer from PS II to PS I (~50% change), (b) the initial distribution of quanta into two pigment systems (~5-20% change), and (c) radiationless thermal transitions in both systems (~3-10% change).

3.6 Analysis 1

3.6.1 A Method for Obtaining the Fraction (α) of Energy Distributed to Photosystem I in a Single Sample

Outlined below is the theoretical basis for obtaining the value of α in a single sample using chlorophyll *a* fluorescence transient and lifetime measurements at 690 and 730 nm. Expressed in terms of photons absorbed per unit time, the fractional photon input to PS I is given by

$$\alpha = \frac{I_1}{I_1 + I_2} \qquad (1A)$$

The transformation from a description of α in terms of theoretical parameters to one by experimentally obtainable quantities requires

greater specificity regarding the exact model assumed for the photochemical apparatus. The model in Fig. 3.1 is followed, but two cases are distinguished (a) the single-spillover model where there is only one donor component for excitation transfer from PS II to PS I, namely, from $\mathcal{2}$ to $\mathcal{1}$ (or, in molecular terms the pathway Chl $\underline{a}_{II}$ → Chl LH → Chl $\underline{a}_{I}$ and Chl $\underline{a}_{II}$ → Chl $\underline{a}_{I}$), and (b) the dual-spillover model where there are two donor complexes for energy transfer: $\mathcal{2}$ to $\mathcal{1}$ and $\mathcal{2}'$ to $\mathcal{1}$ (Chl $\underline{a}_{II}$ → Reaction Center II → Chl $\underline{a}_{I}$).

SINGLE-SPILLOVER MODEL. In the single-spillover model, the key justifications for the transformation of I_1 and I_2 in Eq. 1A to experimental parameters are: (i) that the yield of fluorescence from antenna $\mathcal{1}$ is independent of the state of the set of PS I reaction centers, $\mathcal{1}'$, (Vredenberg and Slooten, 1967) and (ii) the assumption that the fluorescence from $\mathcal{1}$ resulting from quanta transferred from $\mathcal{2}$ consists of two parts: one with constant yield (independent of the state of the PS II reaction centers $\mathcal{2}'$) and the other with variable yield (dependent on the state of $\mathcal{2}'$), and, that the ratio of the variable to constant yield fluorescence from $\mathcal{2}$ (F690) is conserved in this $\mathcal{2}$-sensitized fluorescence from $\mathcal{1}$ (contained in F730)--in other words, the variable fluorescence represents the same fraction of the total fluorescence at 690 nm as for that part of fluorescence at 730 nm excited by quanta initially absorbed by the antenna of PS II, $\mathcal{2}$ (see Butler, 1977). The constant yield part of the F730 signal, $F730_{(0)}$, is composed of the following components: fluorescence excited by quanta initially absorbed by $\mathcal{1}$ ($F_{1(\alpha)}$), fluorescence from $\mathcal{1}$ excited by quanta initially

absorbed by *2* and transferred to *1* ($F_{1(\beta)(0)}$), and fluorescence from the vibrational satellite of the main transition at ~690 nm from *2* ($F_{2(vib)(0)}$); that is,

$$F730_{(0)} = F_{1(0)} + F_{2(vib)(0)} = F_{1(\alpha)} + F_{1(\beta)(0)} + F_{2(vib)(0)} \quad (2A)$$

On the other hand, the variable yield part of the F730 signal, $F730_{(v)}$, consists of two components, that due to energy transfer from *2* to *1* ($F_{1(\beta)(v)}$) and that due to the (vibrational) satellite band of F690 ($F_{2(vib)(v)}$), so that

$$F730_{(v)} = F_{1(v)} + F_{2(vib)(v)} = F_{1(\beta)(v)} + F_{2(vib)(v)} \quad (3A)$$

Subject to the valdity of the interpretations of F730 (see assumption (ii) above), $F_{1(\alpha)}$ is obtained by subtracting from the fluorescence intensity at 730 nm the fluorescence associated with quanta absorbed by *2*. As given by Strasser and Butler (1977b), the expression for $F_{1(\alpha)}$ in terms of experimental parameters is

$$F_{1(\alpha)} = F_{1(M)} - \frac{F_{2(M)}}{F_{2(v)}} \cdot F_{1(v)} \quad (4A)$$

which, by replacing $F_{1(M)}$ and $F_{2(M)}$ by $[F_{1(v)} + F_{1(0)}]$ and $[F_{2(v)} + F_{2(0)}]$, and simplifying, may be written as:

$$F_{1(\alpha)} = F_{1(0)} - \frac{F_{2(0)}}{F_{2(v)}} \cdot F_{1(v)} \quad (5A)$$

where $F_{2(0)} = F690_{(0)}$, $F_{1(M)} = F730_{(M)}$, $F_{2(M)} = F690_{(M)}$,

$F_{1(v)} = [F730_{(M)} - F730_{(O)}]$, and $F_{2(v)} = [F690_{(M)} - F690_{(O)}]$. Also,

$$F_{1(\alpha)} = I_1 \cdot \eta_{F1} \tag{6A}$$

or

$$I_1 = F_{1(\alpha)}/\eta_{F1} \tag{7A}$$

Since $F_{2(M)}$ occurs when $\mathcal{Q}'$ is closed, excitation cycling is maximum and the quanta input to $\mathcal{Q}$ is operationally equal to $I_2 \cdot [(1 - \eta_{T(22')} \eta_{T(2'2)}{}^{-1}]$ (cf. Eq. 3.1c), so that

$$F_{2(M)} = I_2 \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}] \cdot \eta_{F2} \tag{8A}$$

and

$$I_2 = \frac{F_{2(M)}}{\eta_{F2} \cdot [(1 - \eta_{T(22')}\ \eta_{T(2'2)})^{-1}]} \tag{9A}$$

Substituting Eqs. 7A and 9A in Eq. 1A, we obtain

$$\alpha = \frac{\dfrac{F_{1(\alpha)}}{\eta_{F1}}}{\dfrac{F_{1(\alpha)}}{\eta_{F1}} + \dfrac{F_{2(M)}}{\{\eta_{F2} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}]\}}}$$

which, upon multiplication by η_{F1} of both the numerator and the denominator yields:

$$\alpha = \frac{F_{1(\alpha)}}{F_{1(\alpha)} + \{\dfrac{\eta_{F1}}{\eta_{F2} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}]}\} \cdot F_{2(M)}} \tag{10A}$$

By definition, $\eta_{F1} = k_{F1}/\Sigma k_1 = k_{F1} \cdot \tau_{F1(O)}$ (cf. Eqs. 3.1a and 3.2a), and taking the yield of fluorescence from $\mathcal{1}$ to be independent of the state of $\mathcal{1}'$ under normal conditions (see above), $\tau_{F1(O)} = \tau_{F1(M)}$, giving

$$\eta_{F1} = k_{F1} \cdot \tau_{F1(M)}. \tag{11A}$$

Similarly,

$$\eta_{F2} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}] = k_{F2} \cdot \tau_{F2(O)} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}].$$

From Eq. 3.2b,

$$\eta_{F2} \cdot [(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}] = k_{F2} \cdot \tau_{F2(M)}. \tag{12A}$$

Hence, by substituting Eqs. 11A and 12A in Eq. 10A, we have

$$\alpha = \frac{F_{1(\alpha)}}{F_{1(\alpha)} + \frac{k_{F1}}{k_{F2}} \cdot \frac{\tau_{F1(M)}}{\tau_{F2(M)}} \cdot F_2(M)} \cdot \tag{13A}$$

By assuming identical rate constants of fluorescence for chlorophyll _a_ in $\mathcal{1}$ and $\mathcal{2}$, $k_{F1}/k_{F2} = 1$, Eq. 13A becomes

$$\alpha = \frac{F_{1(\alpha)}}{F_{1(\alpha)} + \frac{\tau_{F1(M)}}{\tau_{F2(M)}} \cdot F_{2(M)}}; \tag{14A}$$

β is then calculated as $(1 - \alpha)$ by the condition that $\alpha + \beta = 1$. With these values of α and β, the efficiency of excitation transfer from $\mathcal{2}$ to $\mathcal{1}$ is calculated as described below.

3.6.2 A Method for Calculating the Efficiency of Excitation Transfer from System $\mathcal{2}$ to $\mathcal{1}$

Assuming in Eq. 2A, that $F_{2(vib)(0)} << F_{1(\alpha)} + F_{1(\beta)(0)}$, $F_{1(0)} = F_{1(\alpha)} + F_{1(\beta)(0)}$ (see Strasser and Butler, 1977b), $F_{1(\beta)(0)}$ is given by

$$F_{1(\beta)(0)} = F_{1(0)} - F_{1(\alpha)} \tag{15A}$$

or, alternatively, by substituting Eq. 5A in Eq. 15A:

$$F_{1(\beta)(0)} = \frac{F_{2(0)}}{F_{2(v)}} \quad F_{1(v)} \cdot \tag{16A}$$

In terms of excitation rates, $F_{1(\alpha)}$ is given by Eq. 6A, while

$$F_{1(\beta)(0)} = I_2 \eta_{T(21)} \eta_{F1} = \beta I \eta_{T(21)} \eta_{F1} \tag{17A}$$

so that, by dividing Eq. 6A by Eq. 17A, and equating $I_1 = \alpha I$, we have

$$\frac{F_{1(\alpha)}}{F_{1(\beta)(0)}} = \frac{\alpha}{\beta} \cdot \frac{1}{\eta_{T(21)}}$$

which can be rewritten as:

$$\eta_{T(21)} = \frac{\alpha}{\beta} \cdot \frac{F_{1(\beta)(0)}}{F_{1(\alpha)}} \quad . \tag{18A}$$

Theoretically, Butler and Kitajima (1975a,b) have shown that inclusion of the concept that excitation cycling between $\mathcal{2}$ and $\mathcal{2}'$ occurs when $\mathcal{2}'$ is closed, implies that

$$\frac{F_{2(v)}}{F_{2(M)}} = \eta_{T(22')}\eta_{T(2'2)} \tag{19A}$$

which, upon replacement of $F_{2(v)}$ by $[F_{2(M)} - F_{2(0)}]$ and simplification, leads to the empirical equation for the enhancement factor

$$\frac{F_{2(M)}}{F_{2(0)}} = (1 - \eta_{T(22')}\eta_{T(2'2)})^{-1} \tag{20A}$$

Recalling (Eqs. 3.1b and c) that $\phi_{T(21)(0)} = \eta_{T(21)}$ and that $\phi_{T(21)(M)} = \phi_{T(21)(0)} \cdot (1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}$ we have, by substituting Eq. 20A in Eq. 3.1c,

$$\phi_{T(21)(M)} = \eta_{T(21)} \cdot \frac{F_{2(M)}}{F_{2(0)}} \tag{21A}$$

DUAL-SPILLOVER MODEL. A generalization of the above model is obtained, if, in addition to the pathway for excitation transfer from $\mathcal{2}$ to $\mathcal{1}$, another pathway--from a closed $\mathcal{2}'$ to $\mathcal{1}$--is included. For such a generalization more experimental information on the photochemical apparatus is needed (for unique solutions), in the absence of which additional assumptions must be imposed. From the practical point of view, the major difference of the dual-spillover model from the single-spillover one is that a part of $F_{1(v)}$ must be associated to the $\mathcal{2}'$ to $\mathcal{1}$ transfer process. This leads to a smaller $F_{1(\beta)(0)}$ than in the single-spillover model and, hence, a new set of values for α, $\phi_{T(21)(0)}$, and $\phi_{T(21)(M)}$. Hereafter, in instances when the same parameters in the two models are referred to, pre-superscripts $(^1)$ and $(^2)$ will be used to denote the single- or dual-spillover cases.

One possible course for the development of the dual-spillover model formalism has been outlined by Butler and Kitajima (1975a,b; see also Butler, 1977). In essence the single-spillover formalism is retained and three additional assumptions imposed to facilitate extrapolation of experimental data (see below). The assumptions are: (a) a "reasonable" value for $\eta_{T(22')}$, with the range of admissible values determined from the empirical data, (b) $\eta_{P2'} + \eta_{T(2'2)} + \eta_{T(2'1)} = 1$ subjected to the conditions that $\eta_{P2'} = 1$ when $\mathcal{Q}'$ is open and $\eta_{P2'} = 0$ when $\mathcal{Q}'$ is closed, and (c) $^{(1)}\eta_{T(21)} = {}^{(2)}\eta_{T(21)}$. Using assumption (a), a value for $\eta_{T(2'2)}$ can be estimated from the known value of the product $\eta_{T(22')}\,\eta_{T(2'2)}$ given by the ratio $F_{2(v)}/F_{2(M)}$ (see Eq. 19A). Then, by assumption (b), $\eta_{T(2'1)} = 1 - \eta_{T(2'2)} - \eta_{P2'}$; when $\mathcal{Q}'$ is closed, $\eta_{P2'} = 0$ and drops out. In addition, assumption (b) says that $\eta_{T(2'2)} + \eta_{T(2'1)} \simeq 0$ when $\mathcal{Q}'$ is open because $\eta_{P2'} = 1$, implying that the transfer of excitation energy from $\mathcal{Q}'$ to $\mathcal{L}$ is negligible when the centers are open. When the centers are closed, then $\eta_{T(2'1)}$ is significant and contributes to the variable yield part of fluorescence from $\mathcal{L}$.

The contribution to $^{(2)}F_{1(v)}$ by the $\mathcal{Q} \rightarrow \mathcal{L}$ excitation transfer (Chl $\underline{a}_{II}$ → Chl LH → Chl $\underline{a}_{I}$ or Chl $\underline{a}_{II}$ → Chl $\underline{a}_{I}$) is $I_2\phi_{T(21)(v)}\eta_{F1'}$ where $\phi_{T(21(v)}$ ($= \phi_{T(21)(M)} - \phi_{T(21)(0)}$) is the variable yield of energy transfer from $\mathcal{Q}$ to $\mathcal{L}$. Substituting for $\phi_{T(21)(M)}$ by Eq. 1c, for $(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}$ in the resulting expression by Eq. 20A, and by equating $[F_{2(M)} - F_{2(0)}]$ to $F_{2(v)}$ one obtains the relation

$$\phi_{T(21(v)} = \phi_{T(21)(0)} \cdot \frac{F_{2(v)}}{F_{2(0)}} \tag{22A}$$

or by Eq. 1b as

$$\phi_{T(21(v)} = \eta_{T(21)} \cdot \frac{F_{2(v)}}{F_{2(0)}} \tag{23A}$$

The contribution to $^{(2)}F_{1(v)}$ by excitation transfer from $\mathcal{2}'$ to $\mathcal{1}$ (Chl $\underline{a}_{II}$ → Reaction Center II → Chl $\underline{a}_{I}$) is $I_2\eta_{T(22')}\eta_{T(2'1)}\eta_{F1}$, where the product $\eta_{T(22')}\eta_{T(2'1)}$ is the net efficiency for excitation transfer from $\mathcal{2}$ to $\mathcal{1}$ by the pathway $\mathcal{2} \rightarrow \mathcal{2}' \rightarrow \mathcal{1}$. Thus, in the dual-spillover model the total variable fluorescence from $\mathcal{1}$ is given by the sum of the two contributions described above; that is

$$^{(2)}F_{1(v)} = I_2\ \eta_{F1}[\phi_{T(21)(v)} + \eta_{T(22')}\eta_{T(2'1)}] \tag{24A}$$

This means that the part of $^{(2)}F_{1(v)}$ contributed by the $\mathcal{2} \rightarrow \mathcal{1}$ excitation transfer, labelled as $^{(2)}F_{1(\beta)(v)}$, is given by the relative fraction of $^{(2)}F_{1(v)}$ represented by $I_2\phi_{T(21)(v)}\eta_{F1}$; that is

$$\frac{^{(2)}F_{1(\beta)(v)}}{^{(2)}F_{1(v)}} = \frac{\phi_{T(21)(v)}}{\phi_{T(21)(v)} + \eta_{T(22')}\eta_{T(2'1)}}$$

or

$$^{(2)}F_{1(\beta)(v)} = \frac{\phi_{T(21)(v)}}{\phi_{T(21)(v)} + \eta_{T(22')}\eta_{T(2'1)}} \cdot {}^{(2)}F_{1(v)} \tag{25A}$$

Eq. 25A is the empirical equation for $^{(2)}F_{1(\beta)(v)}$; to calculate $\phi_{T(21)(v)}$, we use Eq. 23A, where $\eta_{T(21)}$ is given by Eq. 18A, as required by

assumption (c); and $F_{2(v)} = F690_{(v)}$ and $F_{2(0)} = F690_{(0)}$; $\eta_{T(22')}$ is obtained by using assumption (a); and $^{(2)}F_{1(v)} = F730_{(v)}$ by assuming $^{(2)}F_{2(vib)(v)} << {}^{(2)}F_{1(v)}$ in Eq. 3A. Then, by the second basic postulate in both the single- and dual-spillover models--that the fluorescence from $\mathcal{1}$ sensitized by $\mathcal{2}$ has the same ratio of variable to constant yield parts as the fluorescence from $\mathcal{2}$ (see discussion of postulate (ii) given under single-spillover model)--

$$\frac{^{(2)}F_{1(\beta)(0)}}{^{(2)}F_{1(\beta)(v)}} = \frac{F_{2(0)}}{F_{2(v)}}$$

or

$$^{(2)}F_{1(\beta)(0)} = {}^{(2)}F_{1(\beta)(v)} \cdot \frac{F_{2(0)}}{F_{2(v)}} \qquad (26A)$$

giving $^{(2)}F_{1(\alpha)} = F_{1(0)} - {}^{(2)}F_{1(\beta)(0)}$, using Eq. 15A. From the empirical values of $F_{2(M)}$, the lifetimes ($\tau_{F1(M)}$ and $\tau_{F2(M)}$) in Table 3.1, and the calculated value of $^{(2)}F_{1(\alpha)}$ from above $^{(2)}\alpha$ can be calculated with Eq. 14A, and $^{(2)}\beta$ is given by $1-{}^{(2)}\alpha$. These newly calculated values of $^{(2)}F_{1(\alpha)}$, $^{(2)}F_{1(\beta)(0)}$, $^{(2)}\alpha$, and $^{(2)}\beta$ then give $^{(2)}\eta_{T(21)}$ by Eq. 18A, from which $^{(2)}\phi_{T(21)(M)}$ is obtained by Eq. 21A.

3.7 Analysis 2

3.7.1 A Critical Survey on Existing Formulations for System Analysis of Chlorophyll a Fluorescence in Thylakoids at 77 K

The first attempt at analyzing chlorophyll fluorescence using a systems level approach was by Butler and Kitajima (1977a,b). This work

provided the basic framework for the more generalized reformulation of the analysis outlined in this chapter. Below are given our critical comments on all the formulations used for studying energy distribution and redistribution in thylakoids in order to clarify the limitations and usefulness of each formulation.

3.7.1.1 Butler and Kitajima Formulation

For the calculation of $\eta_{T(21)}$ by the method developed by Butler and Kitajima (1975a,b), and outlined by Butler (1977), fluorescence intensities from two distinct samples with different excitation distribution and redistribution properties are required along with the following constraints: (1) $\Sigma k_1 = k_{D1} + k_{F1} + k_{T(11')}$ is invariant, (2) $k_{D2} + k_{F2} + k_{T(22')}$ is invariant, and (3) I is invariant. We found that in an experiment with three distinct samples (salt-depleted, Na^+, and $Na + Mg^{2+}$), the three combinations, of two samples each, could give two different sets of values for α and $\eta_{T(21)}$. The cause for this complication is not obvious, but is suggested to originate from the constraints imposed. The measured fluorescence lifetimes at 730 nm, presented in Table 3.1, show that τ_{F1} is relatively constant, providing, for the first time, experimental justification for constraint (1). The validity of constraint (2) cannot be readily tested, but it is questioned by the following argument. Since $\tau_{F2(0)}$ is shown to be constant (see Discussion), the sum of the four rate constants k_{D2}, k_{F2}, $k_{T(21)}$, and $k_{T(22')}$ must be invariant. However, $\eta_{T(21)}$ is variable (see Table 3.4) and since by definition (see Eq. 3.2a)

$\eta_{T(21)} = k_{T(21)}/\Sigma k_2$, $k_{T(21)}$ must be variable if Σk_2 is invariant. This implies that the sum of the remaining terms cannot be invariant contradicting constraint (2), assumed by Butler and Kitajima. The more obvious difficulty results from constraint (3). For a frozen sample with ice crystal formation (see Cho and Govindjee, 1970), the use of directly measured fluorescence signals could introduce serious errors depending upon sample and instrumental geometry. Variations in I, the total photons absorbed per unit time, are difficult to identify and control. However, by averaging numerous sets of signals this source of error could be minimized.

An inspection of the set of equations given by Butler (1977) for calculating α, $\phi_{T(21)(0)}$, and $\phi_{T(21)(M)}$ reveals that the constraints are the consequence of insufficient information on the systems. With only information on fluorescence transients at 77 K, constraint (1) is necessary for obtaining the ratio (R) of rate constants for energy transfer from Chl $\underline{a}_{II}$ to Chl $\underline{a}_{I}$ in the two samples, constraint (2) is necessary for obtaining the formulation for $\phi_{T(21)(0)}$, and constraint (3) is needed in the formulation for α.

3.7.1.2 Strasser and Butler (1977a) Formulation

Advantage is taken here of the fact that the above constraints are dispensable, and that α and $\phi_{T(21)(0)}$ can be calculated for an individual sample, if additional information is available. Strasser and Butler developed equations for α and $\phi_{T(21)(0)}$ using information on the excitation spectra for $F_{(0)}$ and $F_{(M)}$ at 694 and 730 nm, as

well as the absorption spectrum at 77 K. The method is free from assumptions, but is not highly sensitive, and contains tedious calculations which are not readily available without the assistance of a computer. In addition, with the above data, certain information--like the value of $k_{T(21)}$--is not obtainable. It must be cautioned that for all analyses at the system level, the rate constants derived for any system $\mathcal{i}$ ($\mathcal{i}$ = $\mathcal{1}$, $\mathcal{1}'$, $\mathcal{2}$, $\mathcal{2}'$) need not be identical to the properties of individual molecules within the system (see also comments in Butler and Kitajima, 1975b).

Finally, it must be pointed out that the equation for α derived by Strasser and Butler (1977a) and that derived here (Eq. 14A) are alternative representations of Eq. 1A. The differences arise from the choice of expression for I_2. Instead of using Eq. 9A, the Strasser and Butler formulation utilizes Eq. 17A, which may be rewritten as

$$I_2 = \frac{F_{1(\beta)(0)}}{\eta_{T(21)}\eta_{F1}} \tag{27A}$$

That is, by substituting Eqs. 7A and 27A in Eq. 1A one gets

$$\alpha = \frac{\dfrac{F_{1(\alpha)}}{\eta_{F1}}}{\dfrac{F_{1(\alpha)}}{\eta_{F1}} + \dfrac{F_{1(\beta)(0)}}{\eta_{T(21)}\eta_{F1}}}$$

which simplifies to the equation obtained by Strasser and Butler:

$$\alpha = \frac{F_{1(\alpha)}}{F_{1(\alpha)} + \dfrac{1}{\eta_{T(21)}} \cdot F_{1(\beta)(0)}} \tag{28A}$$

3.7.1.3 Harnischfeger's Formula

The equation given by Harnischfeger (1977) for the calculation of α (denoted as α_H) is

$$\alpha_H = \frac{F_{1(\alpha)}}{F_{1(M)} + F_{2(M)}} \quad . \tag{29A}$$

The theoretical basis of Eq. 29A was severely questioned by Butler and Strasser (1977), and it will be shown here on theoretical grounds that Eq. 29A is indeed incorrect and cannot correspond to Eq. 14A (this chapter) or Eq. 28A (Strasser and Butler). Since both Eqs. 14A and 28A originate from Eq. 1A, it will be shown that when Eq. 28A is expressed in theoretical parameters Eq. 1A does not result. Now,

$$F_{1(M)} = F_{1(\alpha)} + F_{1(\beta)(M)} \tag{30A}$$

and

$$F_{1(\beta)(M)} = I_2 \, \phi_{T(21)(M)} \eta_{F1}$$

which upon substitution of $\phi_{T(21)(M)}$ by Eq. 21A, gives

$$F_{1(\beta)(M)} = I_2 \, \eta_{T(21)} (1 - \eta_{T(22')} \eta_{T(2'2)})^{-1} \eta_{F1} \tag{31A}$$

Therefore, by using Eqs. 6A and 31A in Eq. 30A

$$F_{1(M)} = I_1 \eta_{F1} + I_2 \eta_{T(21)} \, \eta_{F1} \, (1 - \eta_{T(22')} \eta_{T(2'2)})^{-1} \tag{32A}$$

Hence, by using Eqs. 8A and 32A in Eq. 29A, one obtains

$$\alpha_H = \frac{I_1 \, \eta_{F1}}{I_1 \eta_{F1} + I_2 (1 - \eta_{T(22')} \eta_{T(2'2)})^{-1} \, [\eta_{T(21)} \, \eta_{F1} + \eta_{F2}]}$$

which simplifies to

$$\alpha_H = \frac{I_1}{I_1 + I_2(1 - \eta_{T(22')}\eta_{T(2'2)})^{-1}\left[\eta_{T(21)} + \frac{\eta_{F2}}{\eta_{F1}}\right]} \tag{33A}$$

Equation 33A is clearly not equivalent to α (cf. with Eqs. 14A and 28A).

CHAPTER 4

FLUORESCENCE, DELAYED LIGHT EMISSION, AND 515 nm ABSORBANCE CHANGE IN THE MICROSECOND TIME SCALE AT ROOM TEMPERATURE

4.1 Introduction

In order to appreciate the measurements presented in this paper, a brief background is given below. The photochemical reaction in PS II may be visualized as (for references, see review by Amesz and Duysens, 1977):

$$Z\ P680\ Q \overset{h\nu}{\rightleftharpoons} Z\ P680^{*}\ Q \overset{(1)}{\rightleftharpoons} Z\ P680^{+}\ Q^{-} \overset{(2)}{\rightleftharpoons} Z^{+}\ P680\ Q^{-} \xrightarrow{(3)} Z^{+}\ P680\ Q \longrightarrow Z\ P680\ Q$$

$$\downarrow h\nu' \qquad (\text{low } \phi_f) \qquad (\text{high } \phi_1) \qquad R\ R^{-} \qquad M\ M^{\cdot}$$

$$D\ P680^{+}\ Q^{-} \overset{(2)}{\rightleftharpoons} D^{+}\ P680\ Q^{-}$$

where Z is an electron donor to the reaction center chlorophyll *a* of photosystem II (P680), Q is an electron acceptor, $h\nu$ is a light quantum, $h\nu'$ is a quantum of delayed light emission, $P680^{*}$ is an excited singlet chlorophyll *a*, $P680^{+}$ is the chlorophyll *a* cation, Q^{-} is a semiquinone anion, R is a secondary electron acceptor (a 2-electron carrier), and M is the charge accumulator involved in oxygen evolution. The initial charge separation (step 1) is very rapid (<20 ns; perhaps < 10 ps, by analogy to photosynthetic bacteria). Step 2 may be as rapid as 30 ns, but could be much slower (μs?) depending on the structural arrangement of Z and P680; in addition, an alternate donor D may donate electrons

to $P680^+$ at a lower rate constant (μs^{-1}) under special conditions. Finally, step 3 may take 100-600 μs. It has been suggested that $P680^+$ and Q are quenchers of chlorophyll a fluorescence, whereas P680 and Q^- are not. Thus, an observed fluorescence yield rise, after a 10 ns flash, may reflect step 2 or its alternative reaction with D, as mentioned above (fluorescence rise due to the decay of another quencher, a triplet, will have to be considered too); the fluorescence decay may reflect step 3 and/or a back-reaction of the charges on the reactants in step 3. Delayed light emission (DLE), which is suggested to originate from the back-reaction of step 1, will be affected by step 2 or its alternative reaction with D (see review by Amesz and Van Gorkom, 1978).

In this chapter, we present data from parallel measurements, in the same chloroplast preparations, of cation-induced changes in the kinetics of fluorescence yield (3-800 μs) and delayed light emission (6-60 μs), and the light saturation curve for delayed light at 100 μs. Other measurements on the light-induced absorbance changes at 515 nm are also presented.

4.2 Materials and Methods

Sample preparation and experimental techniques were as described in Chapter 2. All thylakoid samples were suspended in 100 mM sucrose buffered with 1 mM Tris-HCl to pH 7.2-7.6 at 23 ± 1° C.

ANALYSIS OF DATA. The normalized fluorescence yield at time t ($\Phi_n(t)$) is as calculated by Jursinic et al. (1976):

$$\Phi_n(t) = \frac{\Phi_{F(t)}}{\Phi_{Fo}} = \frac{S(t) - L(t)}{Fo} \qquad (4.1)$$

where Φ_{Fo} and Fo are the fluorescence yield and intensity from the analytic flash without prior actinic flash, and $\Phi_{F(t)}$, S(t) and L(t) are the fluorescence yield, signal intensity (i.e., fluorescence plus delayed light) and delayed light intensity, respectively, at time t.

Fluorescence yield rise kinetics is analyzed according to the exponential relation (see e.g., Jursinic and Govindjee, 1977):

$$\Phi_F(t) - \Phi_{Fo} = (\Phi_M - \Phi_{Fo})\ (1 - e^{-t/\tau}) \qquad (4.2)$$

where $\Phi_F(t)$ and Φ_{Fo} are as defined for Eq. 4.1, Φ_M is the maximum value of $\Phi_{F(t)}$ for a particular exciting flash intensity, and τ is the life-time of fluorescence yield rise--the time when $\Phi_M - \Phi_F(\tau) = e^{-1}\ (\Phi_M - \Phi_{Fo})$. Rewriting Eq. 4.2 as

$$\log\ [\frac{\Phi_M - \Phi_{F(t)}}{\Phi_M - \Phi_{Fo}}] = -\frac{1}{\tau \cdot \ln\ 10}\ t \qquad (4.3)$$

shows that a plot of $\log\ [(\Phi_M - \Phi_F(t))/(\Phi_M - \Phi_{Fo})]$ versus t gives a straight line with a slope $= -(\tau \cdot \ln\ 10)^{-1}$ from which τ can be obtained.

Determination of the effective absorption cross-section of a photosynthetic unit is by an adaptation of the method of Weaver and Weaver (1969) to delayed light measurements. The basic assumption in this method is that the probability of hitting an open photosystem II reaction center during a flash obeys a Poisson distribution. Presented below is a novel approach for analysing a change in the effective absorption cross-section of a photosynthetic unit. Consider the case

when an average of $\sigma \cdot n$ photons hit a photosynthetic unit, where σ is the absorption cross-section of a photosynthetic unit for 337 nm photons, and n is the number of incident photons per flash per cm^2. For generality, let c ($0 \leq c \leq 1$) denote the coupling coefficient for exciton transfer from the antenna to the reaction center; that is, c denotes the probability that a photon absorbed by the antenna will get to the trap. The situation in which $\sigma \cdot n$ photons are absorbed with probability c of being transferred to the trap is effectively the same as when $c \cdot \sigma \cdot n$ photons are absorbed with perfect transfer to the reaction center. In other words, a change in c is operationally the same as a change in the effective absorption coefficient of the pigment array serving the reaction center of photosystem II. The probability (P) that an open reaction center is not closed (i.e., does not undergo charge separation) by a flash is given by $P(0; c\sigma n) = (c\sigma \cdot n)^0 \cdot e^{-c\sigma \cdot n}/0! = e^{-c\sigma \cdot n}$. Therefore, the probability that charge separation occurs at an open reaction center is $1 - P(0; c\sigma n)$ or $(1 - e^{-c\sigma \cdot n})$. Thus, if the intensity of 100 μs delayed light emission is proportional to the probability of occurrence of photochemistry at a reaction center, the flash intensity saturation curve for delayed light (at 100 μs, in our experiments) would be given by the exponential rise according to the equation:

$$L(n) = L_s \ (1 - e^{-c\sigma \cdot n}) \qquad (4.4)$$

where L(n) is the (100 μs) delayed light intensity and L_s is the intensity of delayed light emission at saturation. Equation 4.4 can be written as:

$$\log\left[\frac{L_s - L(n)}{L_s}\right] = -\frac{c\sigma}{\ln 10} \cdot n \qquad (4.5)$$

which gives a linear plot for $\log[(L_s - L(n))/L_s]$ versus n with $L(n) = 0$ at $n = 0$, and a slope $= -(c\sigma/\ln 10)$. The absorption cross-section of a chlorophyll molecule, σ_m, at 337 nm was estimated from optical density measurements, and the amount of sensitization of photosystem II computed as $c\sigma/\sigma_m$. It must be emphasized that with 337 nm excitation an accurate estimate of σ_m in chloroplasts is difficult, as the experimentally obtained optical density at 337 nm must be corrected for absorption by molecules other than chlorophyll. Failure to do so imposes the assumption that only chlorophyll molecules absorb at 337 nm and would lead to an overestimated value for σ_m, and, hence, an underestimated absolute photosynthetic unit size. This error is accentuated if sample scattering is not accounted for in the optical density measurement.

Analysis of the fluorescence yield changes is as follows (see also, Butler and Kitajima, 1975c). At any time, t, the fluorescence yield is given by

$$\Phi_{F(t)} = \frac{k_f}{k_f + k_h + [k_p + (1 - A)k_d]} \qquad (4.6)$$

with

$$k_p = k_T \cdot [T]_t = (k_T \cdot [T]_o) \cdot ([T]_t/[T]_o) = k_{p_o} \cdot A \qquad (4.7)$$

where k_f, k_h, k_p are the rate constants for the depopulation of the first excited singlet state of chlorophyll a by fluorescence,

non-radiative thermal processes, and photochemistry, k_d is the rate constant for energy dissipation by a closed reaction center, k_{p_o} is the maximum value of k_p, k_T is the bimolecular rate constant for energy transfer from the antenna to the reaction center (see Vredenberg and Duysens, 1963; Knox, 1973), $[T]_o$ and $[T]_t$ are the maximum concentration and concentration at any time, t, of open traps, and A is a scaling factor defined as the ratio of $[T]_t$ to $[T]_o$. In a dark adapted sample, prior to an actinic flash, the system is in a state of maximum trapping efficiency, with all traps open, and the fluorescence yield is given by:

$$\Phi_{F_o} = \frac{k_f}{k_f + k_h + k_{p_o}} \tag{4.8}$$

as $A = 1$ and $(1 - A)k_d = 0$. At the peak of the fluorescence yield rise after a saturating flash all the traps are closed, and the yield is:

$$\Phi_{F_M} = \frac{k_f}{k_f + k_h + k_d} \tag{4.9}$$

The equality $k_d = f \cdot k_{p_o}$, where $0 \leq f \leq 1$, may be introduced without loss of generality, since the term k_d in Eq. 9 incorporates the notion that a closed reaction center may still accept and dissipate excitation energy (Butler and Kitajima, 1975c). The ratio, R, of the variable fluorescence yield ($\Phi_{F_M} - \Phi_{F_o}$) to the maximum yield has the following relationship:

$$R = 1 - \frac{\Phi_{F_o}}{\Phi_{F_M}} = \frac{k_{p_o} - k_d}{k_f + k_h + k_{p_o}} = (1 - f) \cdot \Phi_{p_o} \qquad (4.10)$$

where the maximum yield of photochemistry, $\Phi_{p_o} = k_{p_o}/(k_f + k_h + k_{p_o})$. Equations 4.8, 4.9, and 4.10 are independent of the model assumed for the photosynthetic unit, as they can also be derived from the puddle model formulation (see Butler and Kitajima, 1975c). However, when the exciting flash is non-saturating with respect to photochemistry, different relations for the maximum fluorescence yield for the flash, Φ_M, exist for each model. The two extreme cases are: the puddle model, in which a photosynthetic unit consists of one reaction center with no inter-unit energy transfer, and the lake model, in which numerous reaction centers share a common antenna (Robinson, 1967). In the case of the puddle model:

$$\Phi_M = A \cdot \Phi_{F_o} + (1 - A) \cdot \Phi_{F_M}$$

or

$$\frac{\Phi_{F_o}}{\Phi_{F_M}} = \frac{1 - A}{\left(\frac{\Phi_M}{\Phi_{F_o}}\right) - A} \quad . \qquad (4.11)$$

In the case of the lake model: Φ_M is given by Eq. 4.6, and from the Equations 4.6, 4.8, and 4.9, we obtain:

$$\frac{1}{\Phi_{F_M}} = \frac{1}{\Phi_{F_o}} - \frac{1}{1 - A} \cdot \left(\frac{1}{\Phi_{F_o}} - \frac{1}{\Phi_M}\right) \quad .$$

or,

$$\frac{\Phi_{F_o}}{\Phi_{F_M}} = \frac{1 - A \cdot \left(\frac{\Phi_M}{\Phi_{F_o}}\right)}{(1 - A)\left(\frac{\Phi_M}{\Phi_{F_o}}\right)} \quad . \tag{4.12}$$

4.3 Results

4.3.1 Chlorophyll a Fluorescence Yield Rise

The fluorescence yield from 3 to 35 μs after an actinic flash, normalized to 1.0 at zero time, and calculated according to Eq. 4.1, is plotted as a function of time in Fig. 4.1. The close correspondence between the experimental points and the least-squares fit curves (solid lines) according to Eq. 4.2 shows that the fluorescence yield rise is exponential confirming earlier findings for this time range (Mauzerall, 1972). The fluorescence yield, $\Phi_F(t)$, reaches a maximum, Φ_M, between 20 μs and 30 μs after the flash, also in accordance with previous findings (Mauzerall, 1972, 1976; Duysens et al., 1975; Jursinic and Govindjee, 1977). The addition of 5 mM NaCl to a salt-depleted chloroplast sample causes a decrease of ~30% in Φ_M, while the further addition of 5 mM $MgCl_2$ to the NaCl sample causes an increase of ~100%.

A plot of log $[(\Phi_M - \Phi_F(t))/(\Phi_M - \Phi_{F_o})]$ versus t (see Eq. 4.3) is shown in Fig. 4.2. Within the limits of the experimental errors the salt-depleted, 5 mM NaCl replenished, and 5 mM NaCl plus 5 mM $MgCl_2$ replenished samples, from two different batches of chloroplasts, show the same lifetime of rise (τ) of 6.4 ± 0.6 μs.

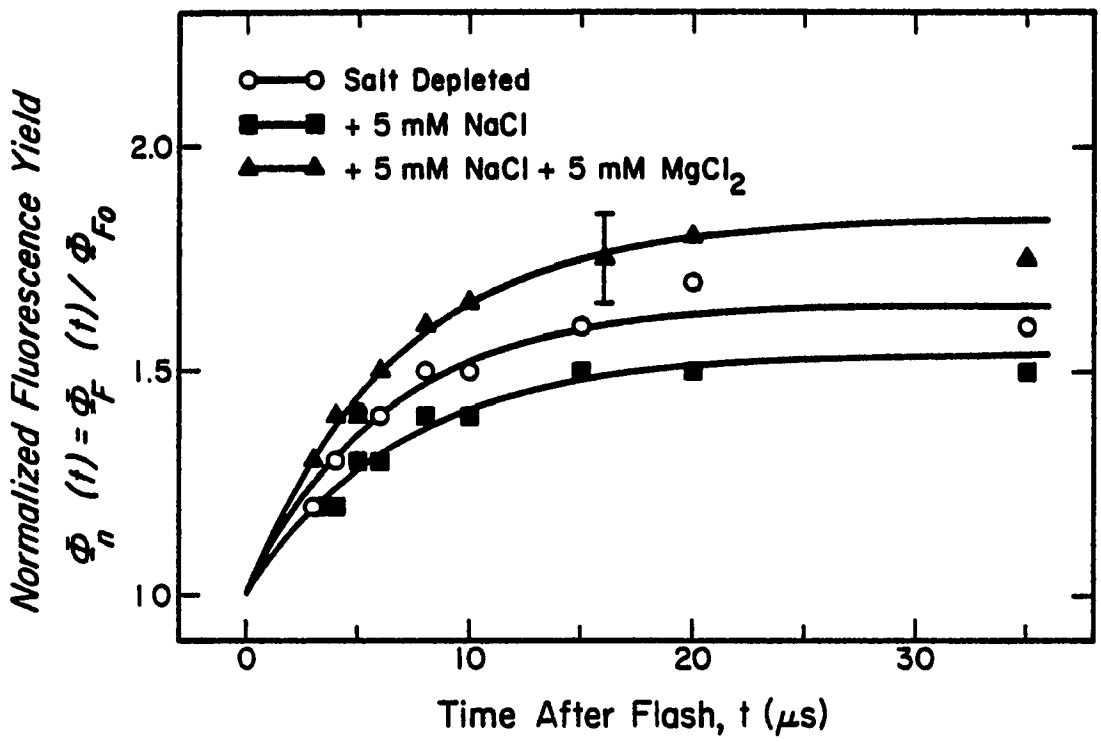

Figure 4.1 Chlorophyll a Fluorescence Yield Rise from 3 to 35 μs, after 10 ns Actinic Flash, Plotted as $\Phi_{F(t)}/\Phi_{F_o}$ versus Time. Points represent experimental data and solid curves are least-squares fit exponential curves according to Eq. 4.2, $\Phi_{F(t)} - \Phi_{F_o} = (\Phi_M - \Phi_{F_o})(1 - e^{-t/\tau})$ with $[\Phi_M/\Phi_{F_o}, \tau(\mu s)] = [1.65, 5.74]$, $[1.54, 6.93]$, and $[1.84, 6.74]$ for the salt-depleted, Na^+ added, and $Na^+ + Mg^{2+}$ added chloroplasts. The Φ_{F_o} values are 2.0 ± 0.1, 1.5 ± 0.1, and 2.5 ± 0.5 for the three cationic conditions. Chlorophyll concentrations were 5 μg/ml and the exciting intensity was 10^{14} incident photons/cm^2-flash. Typical uncertainties are given by the one error bar. For this and the following figures in this chapter, see text for definition of the mathematical symbols.

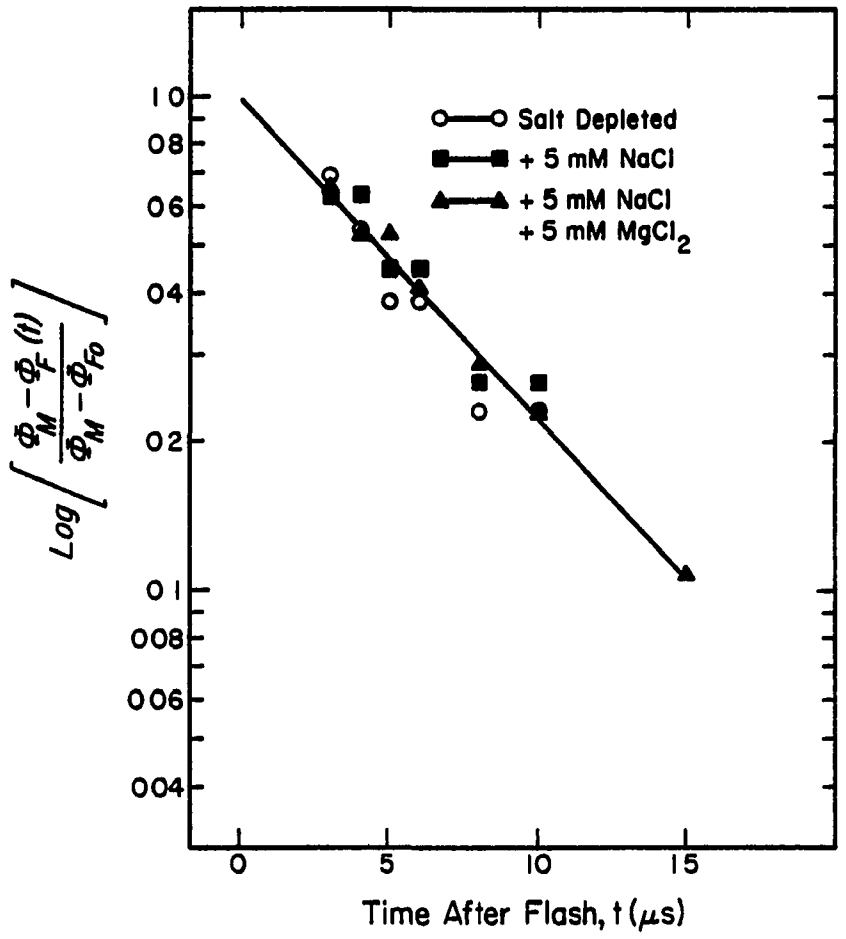

Figure 4.2 Chlorophyll a Fluorescence Yield Rise Data from Fig. 4.1 Plotted as Log $[(\Phi_M - \Phi_{F(t)})/(\Phi_M - \Phi_{F_o})]$ versus Time. The risetime calculated from the slope is 6.7 μs.

4.3.2 Chlorophyll a Fluorescence Yield Decay

The variable fluorescence yield, normalized to Φ_{F_o} values, between 50 and 800 μs for samples in the three cationic conditions are plotted as $[\frac{\Phi_{F(t)}}{\Phi_{F_o}} - 1]$ versus t in Fig. 4.3. The kinetics of this decay is complex (Mauzerall, 1972; Zankel, 1973; Jursinic et al., 1976), and an analytical description of it is as yet unavailable. However, graphical curve fitting shows that our results can be satisfactorily described by a biphasic decay given by the relation: $\frac{\Phi_{F(t)}}{\Phi_{F_o}} - 1 = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, where A_1 and A_2 are the amplitudes of the two exponential phases with lifetimes of τ_1 and τ_2, respectively (Table 4.1). The fraction of fluorescence decaying by the fast phase is changed very slightly from 0.5 to 0.6 upon addition of cations. The most distinct differences are seen in the decrease in τ_1 when cations are added (~90 μs in the absence and ~160 μs in the presence of Na^+ or $Na^+ + Mg^{2+}$), and the decrease in τ_2 when Mg^{2+} is added (~50 ms without Mg^{2+} and ~4 ms with Mg^{2+}). The limited time range of our measurements < 1 ms warrants further investigation on this change in τ_2.

4.3.3 Delayed Light Emission--Intensity and Decay Kinetics from 6 to 60 μs

The intensity of delayed light emission as a function of time from 6 to 60 μs are presented on a semilogarithmic plot in Fig. 4.4. When 5 mM NaCl is added to a salt-depleted thylakoid sample a decrease in the intensity of delayed light by as much as 40% is observed. Addition of 5 mM $MgCl_2$ to a sample with or without 5 mM NaCl causes an enhancement of the delayed light intensity (~2 to 3.5 times that from a sample

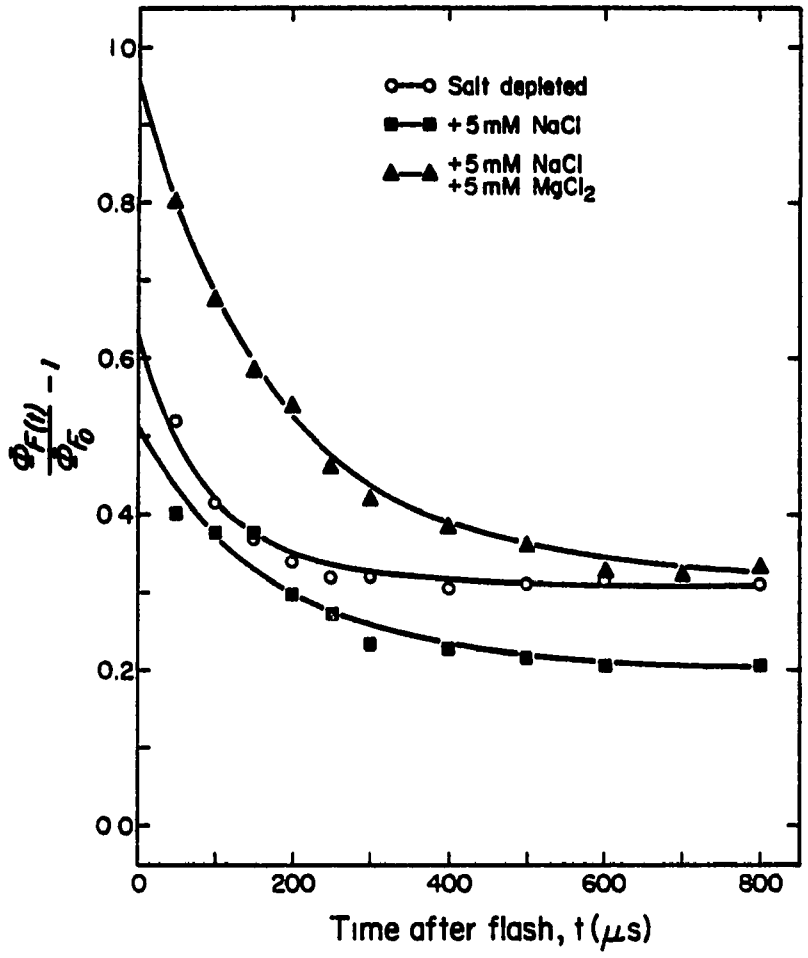

Figure 4.3 Chlorophyll a Fluorescence Yield Decay Plotted as $[(\Phi_{F(t)} / \Phi_{F_o}) - 1]$ versus Time. Solid lines, theoretical curves described by the equation $(\Phi_{F(t)}/\Phi_{F_o}) - 1 = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$ with the values for A_i and τ_i (i = 1,2) given in Table 4.2.

TABLE 4.1

Parameters Describing the Simulated Curve for Variable Fluorescence Yield Decay from 50 to 800 μs

Sample	A(a.u.)	A_1	τ_1 (μs)	A_2	τ_2 (ms)
Salt-depleted	0.624	0.5	91	0.5	50
+5 mM NaCl	0.510	0.6	167	0.4	50
+5 mM NaCl +5 mM $MgCl_2$	0.960	0.6	154	0.4	4

The simulated curve assumes that the fluorescence yield decay to be given by the relation: $\Phi_F/\Phi_{F_o} - 1 = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$, where A_i, τ_i (i = 1,2) are the amplitudes and lifetimes of the two decay phases. In the table, A is the sum of A_1 and A_2 at t = 0 for the data in Fig. 4.3, and A_1 and A_2 are the relative fractions of A represented by the two phases. The best-fit values are within 20% of the reported values. (a.u. stands for arbitrary units.)

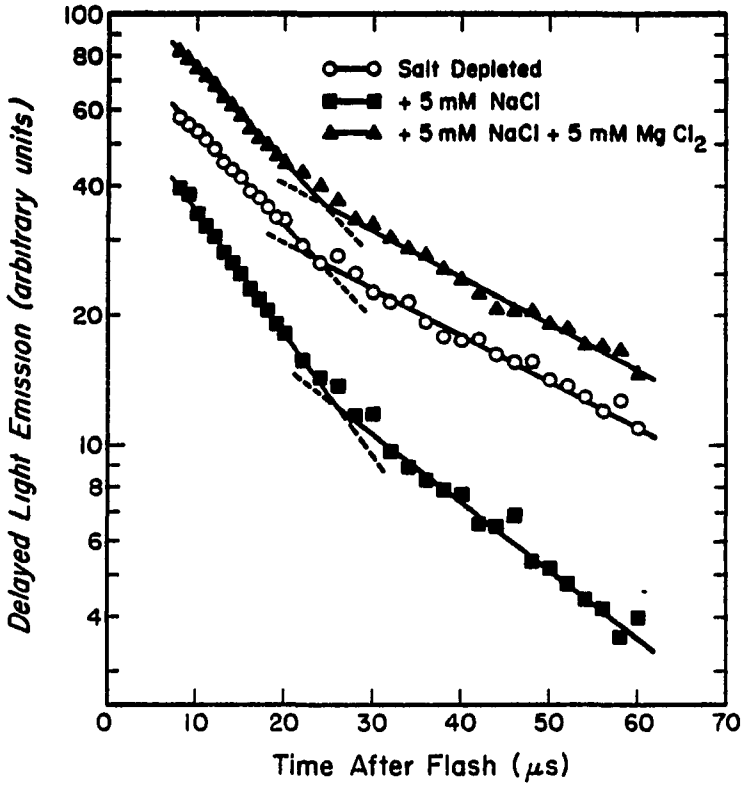

Figure 4.4 Semilogarithmic Plot of Delayed Light Emission Decay against Time. The same samples as in Fig. 4.1 are used.

with 5 mM NaCl).

The delayed light emission between 6 and 60 μs after the flash decays with biphasic kinetics. An analysis of the data by the graphical exponential peeling method (see Van Liew, 1967) is given in Figs. 4.4 and 4.5. Within experimental uncertainties, the salt-depleted, NaCl, and NaCl plus $MgCl_2$ samples show a constant lifetime of 7.2 ± 0.8 μs for the fast phase (Fig. 4.5). A more rigorous analysis by computer curve fitting for a sum of exponentials (Provencher, 1976) gives the results in Table 4.2. Here again, the lifetimes of the decays are relatively constant for the three samples: 8.8-9.6 μs for the fast phase, and 33-40 μs for the slow phase. The proportion of delayed light represented by the fast phase is slightly higher in the sample containing NaCl (76% compared to 58% in salt-depleted and $Na^+ + Mg^{2+}$ samples). We note, however, that the areas under the dalayed light emission curves, calculated as $\Sigma_i \ A_i\tau_i$, and normalized to 1.0 for the $Na^+ + Mg^{2+}$ sample, are 0.74 for the salt-depleted sample and 0.38 for the Na^+ sample. This parallels the values of Φ_{F_M} (see later, Table 4.3).

4.3.4 100 μs Delayed light Emission Light Saturation Curve

The 100 μs delayed light is not saturated at the maximum intensity of our actinic source (Fig. 4.6). The light curve appears to be adequately described by a "single-hit" Poisson saturation, and the saturation intensity of delayed light emission (L_s) for each curve is obtained by iteration using Eq. 4.5. The value of L_s is chosen and a

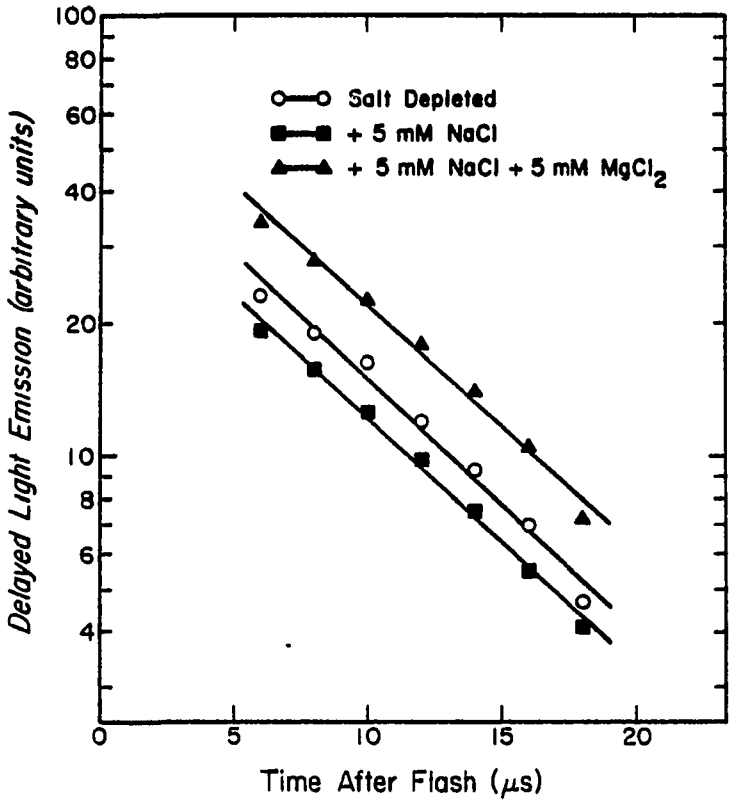

Figure 4.5 Kinetics of Decay of the "Faster" Component of Delayed Light Emission. "Faster" component is obtained by subtracting the "slower" component from the total signal in Fig. 4.4. Solid lines are least-squares fits to the experimental data. The decay times were 7.7 ± 0.1 μs.

TABLE 4.2

Parameters Describing the Best-Fit Curve for Delayed Light Emission from 6 to 60 μs

Sample	L(a.u.)	L_1	τ_1 (μs)	L_2	τ_2 (μs)
Salt-depleted	1013	0.58	9.3	0.42	40.0
+5 mM NaCl	777	0.76	9.6	0.24	32.8
+5 mM NaCl +5 mM $MgCl_2$	1458	0.57	8.8	0.43	36.9

The analysis by the method of Provencher (1976; version 1a March, 1976) for a sum of exponentials is used. In all cases, the best-fit curve ("criterion," $P_{NG} > 0.99$) consists of two components; that is, $L(t) = L_1 e^{-t/\tau 1} + L_2 e^{-t/\tau 2}$, where L(t) is the delayed light intensity at time t, and L_i and τ_i (i = 1,2) are the amplitudes and lifetimes of the two phases. L is the total amplitude of delayed light at t = 0 for the data in Fig. 4.4, and L_1 and L_2 are the relative proportions of the two decaying phases. The uncertainties in the above parameters range from 14 to 50% of the reported values. (a.u. stands for arbitrary units.)

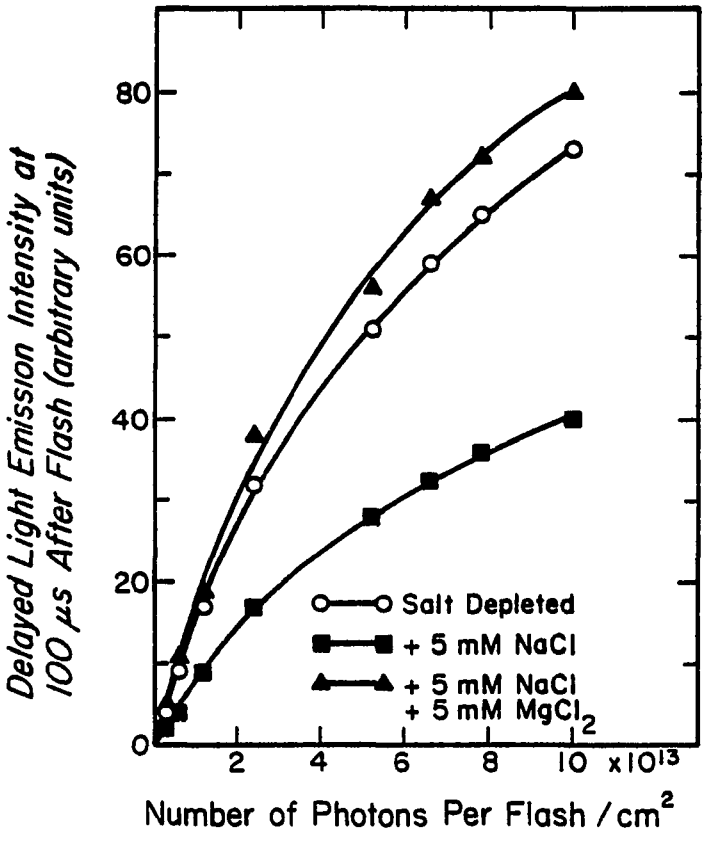

Figure 4.6 Flash-Intensity Saturation Curve for the 100 μs Delayed Light Emission. The curves are plotted as delayed light intensity, L(n), versus the number of incident photons/cm^2-flash, n. At full intensity, n = 10^{14}.

least-squares straight line calculated for the data plotted as log $[(L_s - L(n))/L_s]$ versus n; the value of L_s which satisfies the condition that the least-squares line extrapolated to L(n) = 0 at n = 0 is taken as the saturation value of L(n) for the light curve (Fig. 4.7). The slope of each least-squares line in the plot of log $[(L_s - L(n))/L_s]$ versus n in Fig. 4.7 is proportional to the absorption cross-section of a photosynthetic unit in the samples (see Eq. 4.5). The average calculated values of $c\sigma$ (where c is the coupling coefficient for energy transfer from the antenna to the reaction center and σ is the absorption cross-section) for the salt-depleted, NaCl, and NaCl plus $MgCl_2$ added samples, for two batches of chloroplasts, are 144 ± 3, 136 ± 5, and 163 ± 4, respectively. It must be pointed out that no attempt is made here to remove the assumption imposed by default which leads to an overestimated σ_m, see Analysis of data. However, the relative amounts of sensitization of photosystem II under the three experimental conditions are precise and independent of the value of σ_m, and correspond to ~0.88, 0.83, and 1.0 for the salt-depleted, + Na^+, and + Na^+ + Mg^{2+} samples.

4.3.5 Ratio (R) of Maximum Variable Fluorescence to Total Fluorescence

Since the light saturation curve for 100 μs delayed light emission (Fig. 4.6) indicates that the actinic flash is non-saturating with respect to photochemistry, Φ_{F_M} is calculated for the extreme cases (puddle or lake model) using Eqs. 4.11 or 4.12 (see Table 4.3). The fraction of reaction centers closed, 1-A, by the flash at maximum

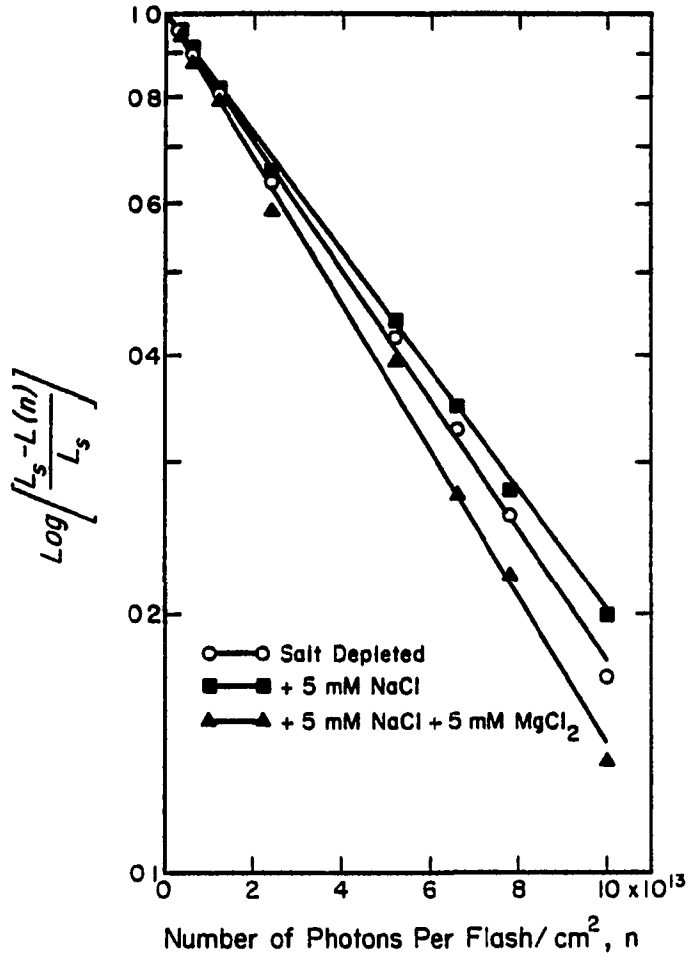

Figure 4.7 Light Saturation Data from Fig. 4.6 Plotted as Log $[(L_s - L(n))/L_s]$ versus n. Solid lines are least-squares fits.

TABLE 4.3

Effects of Na^+ and Mg^{2+} on the Initial and Maximum Relative Yields of Chl a Fluorescence

Sample	$L(n)/L_\infty$	Φ_M/Φ_{F_0}	Φ_{F_M}/Φ_{F_0} [a]Puddle	[b]Lake	Φ_{F_0}	Φ_{F_M} (flash)	Φ_{F_M} (steady state)
Salt-depleted	0.84 ± 0.01	1.50	1.59	1.67	2.0 ± 0.1	0.66 ± 0.03	0.72 ± 0.02
+5 m*M* NaCl	0.81 ± 0.01	1.40	1.49	1.54	1.5 ± 0.1	0.46 ± 0.03	0.38 ± 0.03
+5 m*M* NaCl +5 m*M* $MgCl_2$	0.87 ± 0.01	1.81	1.92	2.04	2.5 ± 0.5	1.00 ± 0.20	1.00 ± 0.03

The fractions of active reaction centers closed by individual flashes, I-A, in column 2 are calculated from the 100 μs delayed light emission flash intensity saturation curve as L(n)/L, for n = 10^{14} incident photons/cm^2-flash. The ratios (Φ_M/Φ_{F_0}) in column 3 are obtained from the fast fluorescence yield rise curves. The quantities (Φ_{F_M}/Φ_{F_0}) in columns 4a and 4b are calculated by the use of Eq. 4.11 for the puddle model, and Eq. 4.12 for the lake model. The initial relative yields, Φ_{F_0} in column 5 are experimentally determined. The maximum relative microsecond fluorescence yields in column 6 are calculated from columns 4a, 4b and 5 and averaged. The maximum relative steady state fluorescence yields at "P" level in column 7 are obtained experimentally. The relative yields in columns 6 and 7 are normalized to 1.00 for the sample containing both NaCl and $MgCl_2$. Results presented are the average values of two separate chloroplast preparations. All experiments were done at 23° C. Notations are as given in the text. Uncertainties denote one standard deviation.

intensity (n = 10^{14} incident photons/flash/cm^2) is obtained as the ratio of the intensity of 100 μs delayed light emission produced by the flash to the saturation value of delayed light emission, L_s, obtained by linear regression. Good agreement is found between the relative maximum yields of microsecond fluorescence (Φ_{F_M}) and the relative yields of "P" level fluorescence in the steady-state (Lavorel, 1959) for the same chloroplast preparations (Table 4.3).

Analysis of the changes in fluorescence yield by the ratio of variable to maximum fluorescence is according to Eq. 4.10. The ratio, R, in Table 4.4 is calculated from the results in Table 4.3 by Eq. 4.10. The cation effects in each set of samples, under our experimental conditions, are relatively independent of the model assumed for the photosynthetic unit. Compared to the salt-depleted control, the sample with 5 mM NaCl shows a lower (12 ± 6%) value for R, while the sample with both 5 mM NaCl and 5 mM $MgCl_2$ present shows an enhanced (30 ± 17%) value. The results in Table 4.4, however, are normalized to 1.00 for R in the sample containing $MgCl_2$.

4.3.6 Light-Induced Absorption Change at 515 nm

The flash-induced absorption change at 515 nm (Δ515) is used as an indicator of the extent of primary charge separation in the P680·Q complex (see Introduction) when the P700 is kept oxidized by 5 mM ferricyanide (see e.g., Witt, 1975; Renger and Wolff, 1975). The results in Fig. 4.8 (a and b) show that the initial amplitude of Δ515, after a single 10 μs flash, is the same for the Na^+ and the $Na^+ + Mg^{2+}$ samples.

TABLE 4.4

Effects of Na^+ and Mg^{2+} on the Calculated Maximum Yield of Primary Photochemistry in a Saturating Flash

$$R = 1 - [\Phi_{F_o}/\Phi_{F_M}] = (1 - f)\cdot\Phi_{P_o}$$

Sample	Puddle Model	Lake Model	R, Relative
Salt-depleted	0.37	0.40	0.77 ± 0.14
+5 mM NaCl	0.33	0.35	0.68 ± 0.18
+5 mM NaCl +5 mM $MgCl_2$	0.48	0.51	1.00 ± 0.05

The quantities $[1 - (\Phi_{F_o}/\Phi_{F_M})]$ which according to Eq. 4.11 give the product of the fractional decrease in the efficiency of excitation energy dissipation by a closed photosystem II reaction center relative to an open one, (1 - f), and Φ_{P_o} are calculated using the results in column 4a and 4b in Table 4.3. The values of R are normalized to 1.00 for the chloroplasts samples to which NaCl and $MgCl_2$ are added. The results presented are the average values for two separate chloroplast preparations. Each uncertainty value presented in the last column represent one standard deviation for the samples averaged and reflect the biological variability. It should be noted that for each sample series R is higher for the salt-depleted sample than for the 5 mM NaCl case.

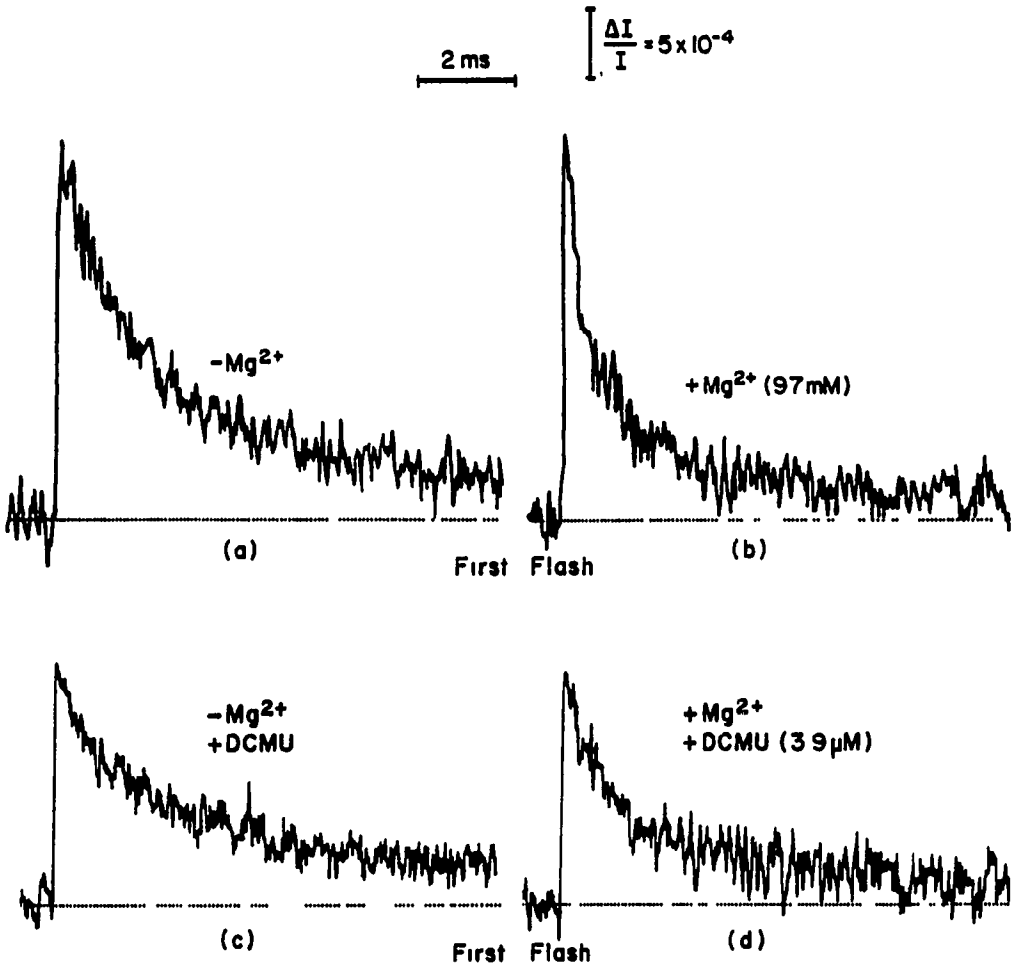

Figure 4.8 Flash-Induced Absorbance Change at 515 nm. Pea thylakoids suspended in 100 mM sucrose + 1 mM Tris-HCl; final pH, 7.2; [Chl] = 30 μg/ml; temperature = 23° C; measuring-beam slit-width = 1 nm. Top traces (a,b) were in the absence of DCMU; lower traces (c,d) were in the presence of DCMU; left column without and right column with Mg^{2+}. All samples contained 9.7 mM NaCl.

An analysis of the recovery of Δ515 by the method of Provencher (1976) gives two components for the Na^+ (A_1 = 59.5%, τ_1 = 1.1 ms; A_2 = 40.5%, τ_2 = 6.4 ms) and $Na^+ + Mg^{2+}$ samples (A_1 = 73.2%, τ_1 = 0.4 ms; A_2 = 26.8%, τ_2 = 3.5 ms), showing that the recovery of the light-induced 515 nm absorbance change is accelerated by Mg^{2+}. Addition of DCMU (Fig. 4-8, a versus c, or, b versus d) results in an ~30% decrease in the initial amplitude of the light-induced Δ515 signal, perhaps the consequence of the lowering of the potential of the couple R/R^- by DCMU, bringing about the reaction $QR^- \rightarrow Q^-R$ (Velthuys and Amesz, 1974; Wollman, 1978). However, Mg^{2+} has no effect on the initial amplitude of Δ515 (Fig. 4.8, c and d), although it slightly enhances the decay rate.

4.4 Discussion

4.4.1 General

The analysis of the cation effects on microsecond fluorescence and delayed light emission, made here, is based on the following concepts (see Butler and Kitajima, 1975c; Butler, 1978): (i) that mono- and di-valent cations affect the degree of coupling of energy transfer between the bulk chlorophyll and reaction center II (see also Chapters 3 and 5), (ii) that a closed reaction center of photosystem II can dissipate excitation energy, (iii) that there is one major source for chlorophyll a fluorescence, and (iv) that the fluorescence yield decay with τ = 100-200 μs reflects the re-oxidation of the stable reduced primary electron acceptor of photosystem II, Q^- (Zankel, 1973).

The observed effects of NaCl and $MgCl_2$ on fluorescence and delayed light emission are not caused by the Cl^- ion, as sodium and magnesium salts with other anions are known to produce the same effects (Murata, 1971; Gross and Hess, 1973; Chapter 2).

4.4.2 Sensitization of Photosystem II

Depending on the coupling coefficient c (see description preceeding Eq. 4.4), which defines the probability for exciton transfer between antenna and reaction center, the conceptual interpretation for the relative amounts of sensitization of reaction center II, P680, could vary. In the limit when c = 1 (perfect coupling) alterations in the degree of sensitization would mean true changes in the absorption cross-section, σ, of the antenna serving P680. On the other hand, if c is allowed to vary ($0 \leq c \leq 1$), changes in the degree of sensitization may occur without changes in σ; that is, the degree of sensitizing of P680 is defined by the degree of coupling for exciton transfer between a constant size antenna with its reaction center. Also, there are no restrictions on simultaneous variations in both c and σ. It is noted that c need not even be restricted to a one-step coupling process. For example, in the tripartite model for chloroplast fluorescence (Butler and Kitajima, 1975a,b; Butler and Strasser, 1977) c would denote the energy transfer coupling between the light-harvesting chlorophyll protein complex (Chl LH) and the photosystem II complex (Chl a_{II}). If it is also proposed that a variable coupling exists between the "bulk" and the reaction center chlorophylls in the photosystem II complex, c

would denote the net coupling between Chl LH and P680; that is, c is the product of the coupling coefficients between Chl LH/Chl a_{II} and Chl a_{II}/P680. Both cases may simply be referred to as the coupling of energy transfer between the antenna chlorophylls and the reaction center. Common usage also refers to this process as the initial partitioning of absorbed quanta to photosystem II. Although the conceptual picture for the sensitization stays undefined, the result (Fig. 4.7) remains that the addition of Na^+ decreases the sensitization of P680 by ~6%, and the subsequent addition of Mg^{2+} then increases the sensitization by ~20% (cf. Butler and Kitajima, 1975a,b; Moya et al., 1977). This result disagrees with the conclusion of Henkin and Sauer (1977) that the major effect of Mg^{2+} ions is to increase the effective absorption cross-section of the pigment array associated with photosystem II photochemistry leading to a 2-fold stimulation in total fluorescence in the presence of DCMU.

4.4.3 Rate Constants for Fluorescence Rise and Delayed Light Decay

The fluorescence yield rise within 35 μs (Fig. 4.1) has been suggested to monitor the rate of disappearance of some fluorescence quencher. The $P680^+$- quencher hypothesis suggests that the quencher is the oxidized reaction center of photosystem II, $P680^+$ (Butler, 1972; Den Haan et al., 1974; Den Haan et al., 1976; Jursinic et al., 1976; Jursinic and Govindjee, 1977), and that the rise of fluorescence reflects the re-reduction of $P680^+$ to P680 by some electron donor, Z or D (see Introduction). In the alternative mechanism, the

carotenoid-triplet-quencher hypothesis (Zankel, 1973; Mauzerall, 1976), the carotenoid triplets with lifetimes 3-4 μs (Chessin et al., 1966; Mathis, 1966; Mathis and Galmiche, 1967; Wolff and Witt, 1969) act as the quencher. The two lifetimes are not the same, possibly because of differences of samples and experimental conditions. Absence of parallel measurements on $P680^+$, carotenoid triplets, and fluorescence rise in the same sample under identical conditions have precluded a choice thus far (see review by Govindjee and Jursinic, 1978). It may be possible that some combination of the two would provide the most satisfactory representation.

Microsecond delayed light emission has been suggested to originate from the back reaction of $P680^+$ with the reduced primary acceptor, Q^- (Van Gorkom and Donze, 1973; see also Lavorel, 1975). This implies that the disappearance of $P680^+$ would lead to a decrease in delayed light emission. The recombination hypothesis and the $P680^+$-quencher hypothesis, taken together, predict that the rate constant of decay of delayed light emission should correspond to that of the rate constant of rise of fluorescence yield. Although such an agreement is observed here--lifetimes of 6.4 ± 0.6 μs for fluorescence rise (Fig. 4.2) and 7.2 ± 0.8 μs for delayed light decay (Fig. 4.5)--it does not constitute a proof. The constancy of these lifetimes in chloroplasts with or without cations added could be interpreted to mean that the rate constant of electron donation from D or Z to $P680^+$ is unaffected by the addition of low concentrations of cations. The $P680^+$-quencher hypothesis is attractive in that it provides the simplest unified

mechanism for fluorescence yield rise and delayed light emission decay, not readily available by the carotenoid-triplet-quencher hypothesis. It is not clear why the disappearance of triplets would lead to a decrease in delayed light emission unless triplets were the source of delayed light (see e.g., Stacy et al., 1971).

4.4.4 The Q^- Decay

Duysens and Sweers (1963) first proposed that the primary electron acceptor, Q, of photosystem II in its oxidized state is a quencher of chlorophyll a fluorescence. In continuous light experiments, at the onset of illumination Q is in its oxidized state and the fluorescence yield is low; with prolonged illumination Q is reduced to Q^- and the fluorescence yield is high, giving the "P" level of fluorescence (Lavorel, 1959; Govindjee and Papageorgiou, 1971). Extending this hypothesis to flash excitation experiments, the maximum fluorescence yield state 20-30 μs after a short saturating flash is assumed to be one in which all Q's are in the Q^- state (equivalent to the "P" level). This suggestion is supported by our observation that the maximum fluorescence yields at ~30 μs for chloroplasts in the three cationic conditions closely matched their relative "P" level yields (last two columns in Table 4.3). This suggestion is also consistent with the interpretation of the fluorescence yield decay. The kinetics of decay is biphasic over the interval of interest here (Mauzerall, 1972; Zankel, 1973; Jursinic et al., 1976), the initial phase decays with an amplitude (relative to the total variable fluorescence) and half-time ($t_{\frac{1}{2}}$) which differs slightly between the two previous reports:

2/3 and ~200 μs according to Zankel (1973) and 3/4 and ~170 μs according to Mauzerall (1972). Based on the findings that the addition of DCMU eliminates this phase of the decay (Zankel, 1973) and that lowering the temperature from 25° to 5° C greatly diminished its amplitude Mauzerall, 1972), it was suggested that the fast phase of the fluorescence yield decay reflects the re-oxidation of Q^-. The fluorescence yield decay for the salt-depleted sample has a $t_{\frac{1}{2}}$ about one-half that of chloroplasts with 5 mM NaCl, but the subsequent addition of 5 mM $MgCl_2$ produces no further change (see Table 4.1). This suggests that the rate constant of Q^- re-oxidation in the absence of added cations is larger than in the presence of low concentrations of mono- and di-valent cations. The slow phase with $t_{\frac{1}{2}}$ in milliseconds probably reflects the equilibrium between Q^-, the connector molecule, R (Bouges-Bocquet, 1973; Velthuys and Amesz, 1974; Diner, 1975), and the plastoquinone pool. Although the addition of Mg^{2+} may appear to accelerate this decay by about an order of magnitude (Table 4.1), we emphasize again that the time range over which the decay was measured precludes any definitive conclusion about the lifetime (τ_2) of this slow phase.

4.4.5 Amplitude of 6 to 100 μs Delayed Light Emission

Assuming the delayed light emission in this time scale originates from the back reaction between $P680^+$ and Q^- (Van Gorkom and Donze, 1973; Govindjee and Jursinic, 1978), a change in its amplitude could be the consequence of one or more of the following causes: (1) a change in the quantum yield (ϕ_L) of delayed light emission, where $\phi_L = L/J$, L being the intensity of delayed light emission, and J the rate of production of excited state Chl, (2) a change in the rate constant for

recombination of $P680^+$ and Q^-, and (3) a change in the concentration of $P680^+$ and/or Q^-.

Figures 4.4 and 4.6 show that the intensities of delayed light, L, between 6 and 100 μs in the order of their magnitudes are $L(Na^+) < L(\text{salt-depleted}) < L(Na^+ + Mg^{2+})$. The intensity of delayed light from chloroplasts with low concentrations of NaCl and $MgCl_2$ was from 2 to 3.5 fold greater than the intensity in samples containing only NaCl. Barber et al. (1977) have shown that the change in intensity of millisecond delayed light follows qualitatively the change in fluorescence yield induced by mono- and di-valent cations. Since an approximately 1.5 fold difference in fluorescence yield exists between the Na^+ and the $Na^+ + Mg^{2+}$ samples throughout the microsecond time range (see Figs. 4.1 and 4.3) a parallel change in the quantum yield for microsecond delayed light cannot be disregarded without additional information (also, P. Joliot, personal communication).

If, in the recombination and emission process, the delayed light photon is emitted from the vicinity of an open reaction center with yield close to Φ_{F_o} (fluorescence yield when all traps are open), then changes in delayed light will have to be due to changes in the concentration of the precursors ($P680^+$ and Q^-) or the rate constant of their recombination. Theoretically a Mg^{2+}-induced increase in the former, starting at 6 μs after the flash, can result from one of the two causes: (a) an increase in the initial production of $[P680^+]$ and $[Q^-]$ by the flash--the idea that Mg^{2+} ions somehow cause an increase in the number of reaction centers capable of photochemistry (see e.g., Li, 1975; Rurainski and Mader, 1977; Bose and Arntzen, 1978), and (b) a

Mg^{2+}-induced decrease in the rate constant for electron donation to $P680^+$ by its primary donor Z, without change in any of the other parameters affecting delayed light. Case (a) is ruled out by the results of the 515 nm measurements (Fig. 4.8), and by the preliminary observations of T. Wydrzynski in P. Mathis' laboratory, that cations have no significant effect on the amplitude of X-320 (a monitor of the primary electron acceptor Q). Case (b) must be considered because direct monitoring of the kinetics of [$P680^+$] changes by Van Best and Mathis (1978) suggests that the half-time for the electron transfer is about 25-45 ns. Direct measurements of the cation effects on [$P680^+$] in this time scale is not yet available.

4.4.6 Primary Charge Separation and the 515 nm Absorbance Change

The constant initial amplitude of the Δ515 signal (Fig. 4.8, a and b, or, c and d), after a single flash, demonstrates that no Mg^{2+}-induced enhancement of primary charge separation exists in these thylakoid preparations. The Mg^{2+}-enhanced rate of recovery of the Δ515 signal may indicate an enhancement in the rate of re-oxidation of Q^- by Mg^{2+}.

4.4.7 Radiationless De-Excitation of Singlet Excited Chlorophyll

Analysed according to Eq. 4.10, the ratio, R, of the variable to maximum fluorescence yield is defined by the product of two terms: (1 - f), the fractional decrease in the efficiency of excitation energy dissipation by a closed photosystem II reaction center compared to an open one, and Φ_{p_o}, the yield of primary photochemistry. A re-examination of Eqs. 4.7 and 4.10 shows that R is defined by five

parameters: k_f, the rate constant for fluorescence; k_h, the sum of rate constants for all radiationless events in the bulk chlorophyll; k_d, the net rate constant for radiationless transitions in the closed reaction center; k_T, the rate constant for energy transfer to the reaction center; and $[T]_o$, the maximum concentration of open traps in the sample. For our discussion it will be assumed that k_f is constant for the three different samples because of the close resemblance of their absorption and emission spectra at room temperature (see also Malkin and Siderer, 1974 and Fig. 2.1). k_T is directly related to c, the coupling coefficient for exciton transfer from the antenna to the trap. The small variations (<20%) in the sensitization of photosystem II suggest that changes in k_T are small. In the present discussion, a brief survey of the consequences of changes in the remaining parameters is made, which will be used later. With reference to Eqs. 4.7-4.10, a change in k_h would affect Φ_{P_o}, Φ_{F_o}, and Φ_{F_M}, a change in k_d would affect f and Φ_{F_M}, and a change in $[T]_o$ would affect f, Φ_{P_o}, Φ_{F_o}, and Φ_{F_M}.

Referring again to Eq. 4.10, one extreme possibility assumes that Φ_{P_o} is constant for all the samples, and any variations in R result from changes in f. In this case, the decrease in R with addition of Na^+ implies an increase in f; that is, Na^+ increases the efficiency for radiationless transitions at a closed reaction center. The process is reversed when Mg^{2+} is added subsequent to Na^+. The other extreme case assumes that (1 - f) is constant, and any differences in R comes about because of differences in Φ_{P_o}, so that $R(Na^+) < R(Na^+ + Mg^{2+})$ implies

that $k_h(Na^+) > k_h(Na^+ + Mg^{2+})$. In other words, Na^+ increases the rate constant, and, hence, the efficiency for some radiationless transition in the bulk chlorophyll of photosystem II, and Mg^{2+} reverses this change. Finally, a change in both f and Φ_{P_o} brought about by a change in $[T]_o$ is considered unlikely in view of our Δ515 results. A brief comment should be made on the implications of changes in k_h and k_d. Possible radiationless transition pathways in both processes include intersystem crossing to the triplet manifold and internal conversion. In addition, k_h includes the radiationless transfer of energy from the bulk chlorophyll molecules either in the light-harvesting complex or the photosystem II complex to the photosystem I, while k_d includes the direct transfer of energy from a closed reaction center II (perhaps in the state $P680 \cdot Q^-$) to the photosystem I (cf. Butler, 1978).

Based only on the data in this chapter, no definitive conclusion can be reached regarding the changes in k_h and k_d. An estimate of the absolute values of the rate constants of the radiationless processes $(k_h + k_d)$ is made using results of chlorophyll *a* fluorescence lifetimes at room temperature. At pH 7.2, the P-level fluorescence lifetimes for the Na^+ and $Na^+ + Mg^{2+}$ samples of thylakoids are 1.0 ± 0.1 and 2.0 ± 0.1 ns (given later in Chapter 6, Table 6.6). Using an intrinsic lifetime ($\tau_o = k_f^{-1}$) of 15.2 ns for chlorophyll *a* fluorescence (Brody and Rabinowitch, 1957) and Eq. 4.9, the ratio $(k_h + k_d)/k_f$ for the Na^+ and the $Na^+ + Mg^{2+}$ samples are 14.2 and 6.6. This analysis implies a 2-fold decrease in $(k_h + k_d)$ upon addition of Mg^{2+} to a Na^+ sample.

Using superscripts "-" and "+" to denote the Na^+ and $Na^+ + Mg^{2+}$ samples, respectively, Eq. 4.10 may be written as:

$$\frac{R^+}{R^-} = \frac{1 - f^+}{1 - f^-} \cdot \frac{k^+_{p_o}}{k^-_{p_o}} \cdot \frac{\tau^+_{F_o}}{\tau^-_{F_o}} \qquad (4.13)$$

where the 0-level fluorescence lifetime, $\tau_{F_o} = (k_f + k_h + k_{p_o})^{-1}$. From the knowledge the τ varies linearly with F for the 0 to P transient (Briantais et al., 1972, 1973; Moya et al., 1977), τ_{F_o} can be calculated as the product of the P-level lifetime and the ratio F_o/F_M (reciprocal of column 4, Table 4.3). Since by Eq. 4.7, $k_{p_o} = k_T \cdot [T]_o$, and variations in k_T are small <20% (see above) and $[T]_o$ = constant (from Δ515 results), k_{p_o} is constant to within 20%. Substituting for the values of R^+, R^- (using Table 4.4), $\tau^-_{F_o}$ and $\tau^+_{F_o}$ (using the respective values of τ_{F_M} and F_o/F_M), it is found that $1 - f^-/1 - f^+ = 1.07$, showing that variations in f between the samples are small. Hence, it is concluded that most of the changes in $(k_h + k_d)$ represent changes in k_h, the rate constant for radiationless events in the PS II antenna--corresponding to changes in k_{D2}, $k_{T(21)}$, and $k_{T(22')}$ in Chapter 3.

4.5 Concluding Remarks

New results are presented in this chapter on the effects of mono- and di-valent cations on concurrent changes in the microsecond yields and kinetics of chlorophyll a fluorescence and delayed light emission, and the light saturation curve for the latter at 100 μs, following a 10 ns flash at 337 nm. (1) The fluorescence yield increases exponentially from 3 to 30 μs (lifetime, τ, 6.4 ± 0.6 μs), and decays biphasically between 50 and 800 μs. (2) The delayed light emission

decays biphasically with two exponential phases: fast phase, τ = 7-10 μs, and slow phase, τ = 33-40 μs. (3) The light saturation curve for 100 μs delayed light emission is satisfactorily represented by a one-hit Poisson saturation curve. (4) Addition of 5 mM NaCl to salt-depleted chloroplasts decreases (by as much as 40%) the yields of microsecond fluorescence and delayed light emission, and the subsequent addition of 5 mM $MgCl_2$ increases the yields ($\geq$ 2x over samples with only NaCl). (5) The fluorescence yield rise and delayed light emission decay kinetics are independent of low concentrations of cations. The lifetime of the fast phase of fluorescence decay changes from ~90 μs to ~160 μs, when Na^+ or $Na^+ + Mg^{2+}$ are added. (6) The light-induced absorbance change at 515 nm is the same in the presence or absence of Mg^{2+}, but the recovery is enhanced by Mg^{2+}.

Based on a detailed analysis presented in this chapter, the following conclusions regarding the effects of low concentrations (few mM) of mono- and di-valent cations in sucrose-washed chloroplasts at room temperature are made: (a) Na^+ decreases (~6%) and Mg^{2+} increases (~20% compared with the Na^+ sample) the sensitization of photosystem II photochemistry; this effect is small, but significant. (b) Mg^{2+} decreases the efficiency for radiationless transitions in singlet excited chlorophyll *a* in the antenna of photosystem II; this includes non-radiative energy transfer to photosystem I ($k_{T(21)}$ in Chapter 3), intramolecular intersystem crossing and internal conversion (collectively denoted by k_{D2} in Chapter 3). The ratio of the sum of rate constants for radiationless transitions to that for fluorescence

decreases by ~2-fold upon the addition of Mg^{2+} to a sample containing Na^{+}. (c) Mg^{2+} does not increase the total number of PS II reaction centers capable of primary photochemistry. (d) The rate constant for re-oxidation of Q^{-} decreases (about 50%) in the presence of Na^{+} or $Na^{+} + Mg^{2+}$. These conclusions imply that cations produce multiple changes in the primary photoprocesses of photosystem II at physiological temperatures. It is proposed that these changes co-exist and are mutually independent.

The present analysis of microsecond delayed light emission data has brought into focus the difficulties in their interpretation without parallel measurements on the formation and the relaxation of the oxidized reaction center chlorophyll *a* $P680^{+}$ and its reduced primary electron acceptor Q^{-}. In spite of these difficulties, delayed light emission can be used for calculating the photosensitization of pigment system II photochemistry as shown here by the analysis of the 100 μs delayed light emission saturation curve. Although somewhat similar difficulties exist in the interpretation of the chlorophyll *a* fluorescence yield data in the microsecond range, its analysis leads to the conclusion that large changes in the rate constants for radiationless transitions exist.

CHAPTER 5

FLUORESCENCE POLARIZATION CHANGES AND ENERGY TRANSFER AT ROOM TEMPERATURE

5.1 Introduction

In the preceeding chapters we have seen the mono- and di-valent cations concurrently affect several primary photoprocesses in thylakoids (see Chapter 1 for a general introduction), with the largest effect on the excitation energy redistribution from PS II to PS I (Butler and Kitajima, 1975a; Chapter 3). It is generally accepted that excitation energy transfer in the photosynthetic system is by Förster's inductive resonance mechanism (Duysens, 1964; Knox, 1975, 1977) in which the pair-wise transfer rate is dependent upon the distance ($\propto r^{-6}$, where r is the distance), the orientation factor, $\kappa^2 (\kappa^2 \leq 4)$, between the donor and acceptor molecules, and the overlap of the donor fluorescence with the acceptor absorption spectrum (Förster, 1965, 1967). Since the degree of polarization of fluorescence is an indicator of the extent of excitation energy migration and/or of the orientation of the molecules (Knox, 1975; Michel-Villaz, 1976), we have measured, at room temperature, the effects of cations on chlorophyll a (Chl a) fluorescence polarization (a) at wavelengths selected to monitor preferentially PS II or PS I emission, and (b) at 730 and 762 nm (to avoid artifacts due to scattering of excitation light) as function of excitation wavelength. Supporting results from the effects of DCMU and m-dinitrobenzene (DNB), of blue excitation,

and of the influence of cation concentration, bulk temperature, and the level of the fluorescence transient on the fluorescence polarization are also reported.

5.2 Materials and Methods

The methods of sample preparation and the experimental techniques were as given in Chapter 2. Ethanol was used in the preparation of stock solutions of DCMU (1 mM) and DNB (165 mM). With respect to the total volume, the ethanol content was <0.5% or ≤2% in any experiment in which DCMU or DNB, respectively, were used. It was independently confirmed that even when the ethanol content was 2%, no significant effects on fluorescence polarization were observed. All experiments reported were in the pH range 7.0-7.5, and temperature range 21-26° C. Other details are given in the legends of the tables and figures.

5.3 Results

5.3.1 Energy Transfer Increase and Chlorophyll a Fluorescence Polarization Decrease: The DCMU Effect

Mar and Govindjee (1972) discovered that the addition of DCMU to algae decreases the degree of polarization (P) of its steady-state chlorophyll a fluorescence (F). By correlating this effect of DCMU with that of the compound in increasing the fluorescence emission--this is also true for the fluorescence lifetime (Müller et al., 1969)--it was proposed that the depolarization (decrease in the degree of polarization) may reflect an increase in the extent of energy migration when

energy trapping is decreased by closure of the PS II reaction centers. This DCMU effect has since been confirmed by Whitmarsh and Levine (1974) and Becker et al. (1976) for algae, as well as for isolated chloroplasts. Becker et al. (1976) have further shown that the DCMU effect exists only for excitation below 680 nm. This effect of DCMU is also confirmed here for pea chloroplasts prepared by the method described by Van Ransen et al. (1978) (Table 5.1). Blue and red actinic light were used to demonstrate the independence of the effect on excitation wavelengths below 680 nm--for both 460 and 660 nm excitation, 1 μM DCMU causes an ~21% decrease in the degree of polarization of fluorescence at 686 nm at room temperature. Similar effects were observed for sucrose-washed thylakoids.

5.3.2 Energy Transfer Decrease and Chlorophyll a Fluorescence Polarization Increase: The m-Dinitrobenzene Effect

The addition of m-dinitrobenzene (DNB) produces an effect opposite to that of DCMU. This compound quenches the fluorescence intensity and, within certain limits of quenching, changes the kinetics of the photochemical O to P rise in the millisecond fluorescence transient from sigmoidal to exponential (Lavorel and Joliot, 1972). The kinetics change has been attributed to the decrease of cooperativity between the photosynthetic units because of the interruption of energy transfer across the boundaries between these units by non-photochemical energy traps formed by DNB (Lavorel and Joliot, 1972). We present in Fig. 5.1 the effects of varying the DNB concentration on the steady-state

TABLE 5.1

DCMU Effect on the Degree of Polarization of Chlorophyll *a* Fluorescence at 686 nm

	Degree of Polarization (%)	
	Excitation Wavelength	
Sample	460 nm	660 nm
Control	2.8 ± 0.1	3.6 ± 0.1
+ 1 μM DCMU	2.2 ± 0.1	2.9 ± 0.1

Samples consisted of 3 ml suspensions of thylakoids ([Chl] = 10 μg/ml) in 50 mM tricine-NaOH (pH 7.6), 300 mM sorbitol, and 5 mM $MgCl_2$. Fluorescence was detected through a Schott RG 665 glass filter and an interference filter at 686 nm (FWHM, 6.4 nm).

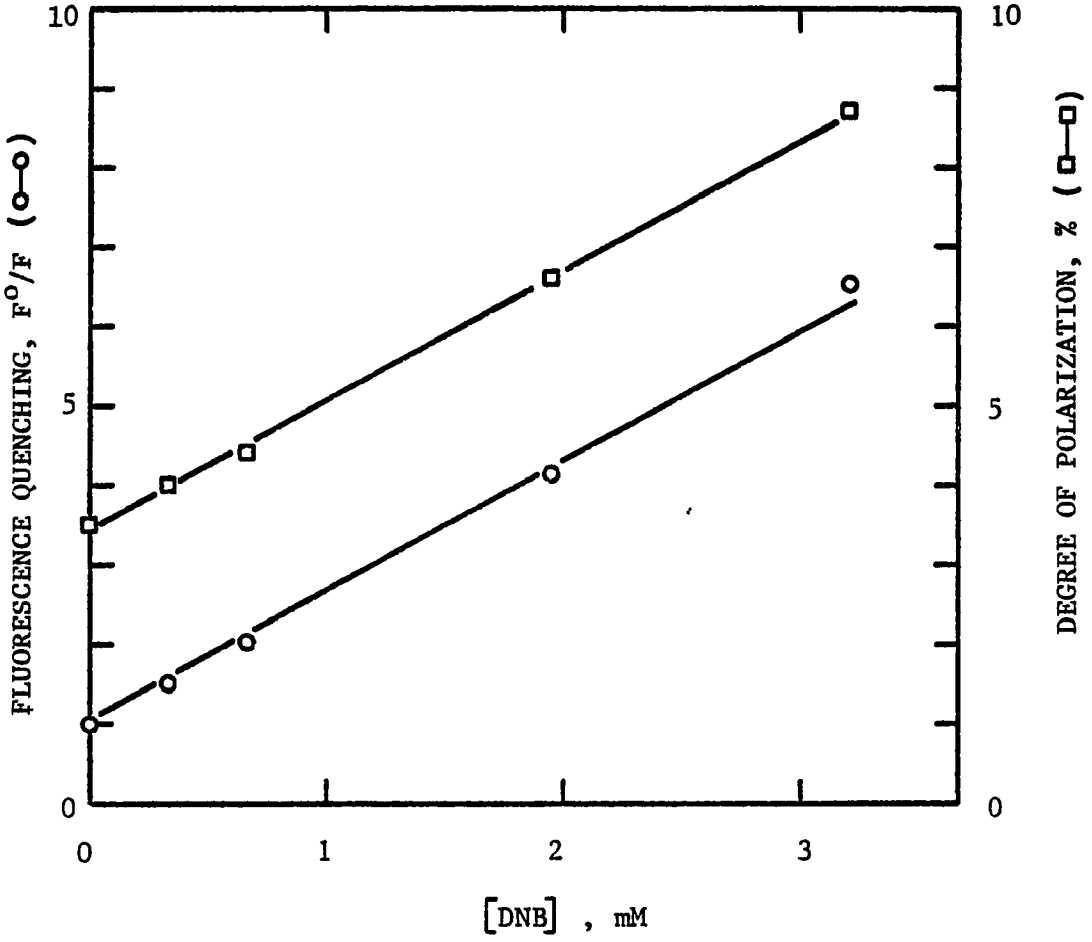

Figure 5.1 Effect of m-Dinitrobenzene on Fluorescence Intensity and Degree of Polarization. Excitation at 480 nm (band-pass, 10 nm); fluorescence detected through Schott RG 665 glass filter; chlorophyll concentration, 5 µg/ml; DCMU, 5 µM; temperature, 26° C.

fluorescence intensity and polarization from salt-depleted thylakoids in the presence of DCMU. It is seen that with excitation at 480 nm both the extent of quenching of total fluorescence (measured by the ratio of the fluorescence intensity in the absence of DNB to that in its presence, F^o/F, and its degree of polarization varies linearly with the concentration of DNB under our experimental conditions, implying a linear dependence between the two fluorescence parameters. This property is further illustrated for excitation at 600 nm in Fig. 5.2 for the fluorescence observed at 680 nm (interference filter, I.F.; FWHM, 14 nm) and at longer wavelengths through a glass cut-off filter (RG 10, transmission <1% at 694 nm). Hereafter, the wavelength corresponding to 1% transmission will be referred to as the cut-off point.

5.3.3 Cation Effects on Fluorescence Polarization

The addition of Na^+ (~5 mM) to salt-depleted thylakoids results in a concurrent increase in the degree of polarization and a decrease in its relative intensity when F > 650 nm (RG 665), F680 (680 nm I.F.; FWHM, 14 nm), or F > 694 nm (RG 10) is measured (Table 5.2). Subsequent addition of Mg^{2+} results in a reversal of the Na^+ effects. The small magnitude of these changes prompted us to search for more favorable experimental conditions for their observation. The results in Table 5.2 and 5.3 represent some of the conditions attempted. It is noted that the Mg^{2+}-induced decrease in the degree of polarization is observed for excitation with blue or red light. The Na^+ effect is sometimes absent when blue excitation is used. The implication of

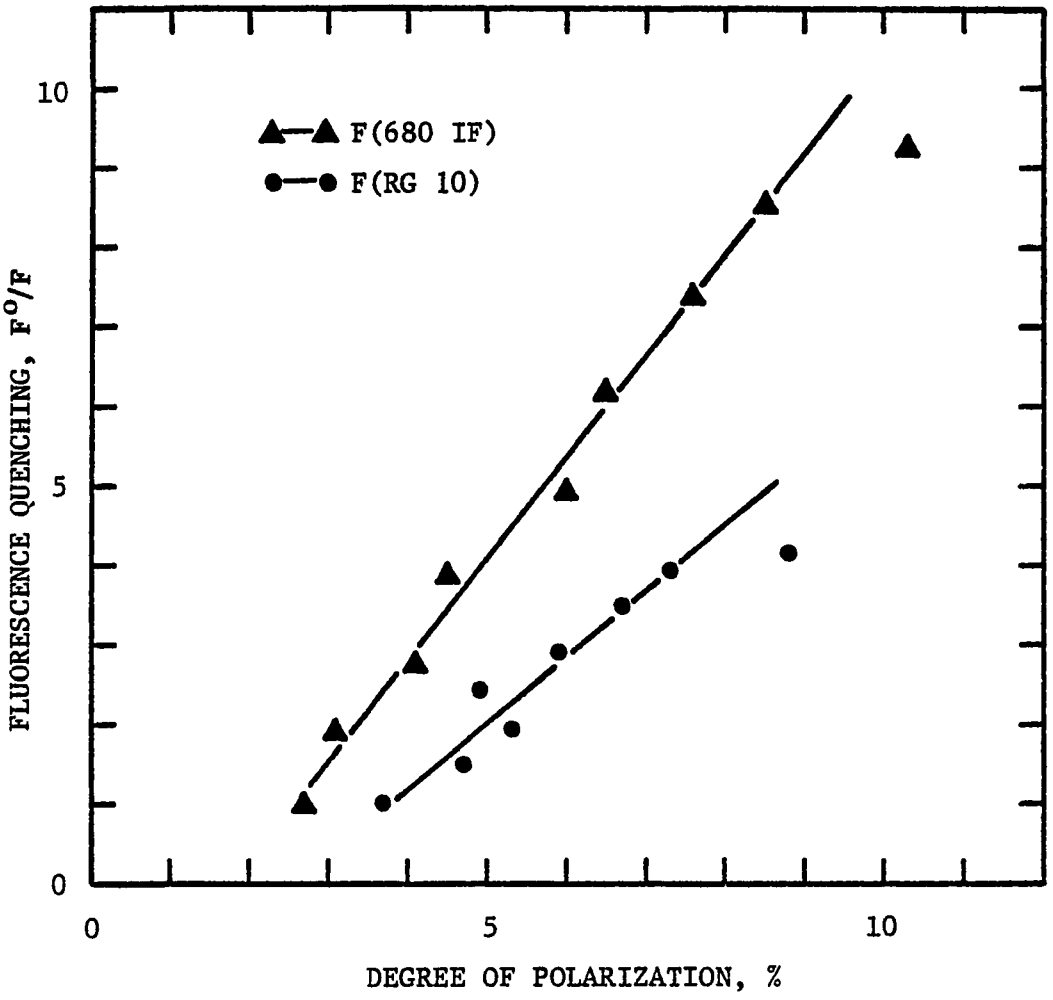

Figure 5.2 Linear Relation between Extent of Quenching and Degree of Polarization of Fluorescence. Excitation at 600 nm (band-pass, 8.3 nm); fluorescence detected through Schott RG 10 glass filter or interference filter at 680 nm (FWHM, 14 nm); chlorophyll concentration, 5 µg/ml; temperature, 23° C.

TABLE 5.2

Cation Effects on the Degree of Polarization of Fluorescence Observed Through Different Red Filters

Treatment	Degree of Polarization (%)		
	RG 665 (F > 650)	680 I.F. (F680)	RG 10 (F > 694)
Salt-depleted	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.1
3 mM NaCl	3.1 ± 0.3	3.0 ± 0.4	3.4 ± 0.4
3 mM NaCl 3 mM $MgCl_2$	2.3 ± 0.2	2.2 ± 0.2	2.6 ± 0.2

Fluorescence was excited at 600 ± 8.3 nm, and detected through a Schott RG 665, RG 10, or an interference filter (I.F.) at 680 nm (FWHM, 14 nm). For the glass cut-off filters, the numbers given in parenthesis denote the approximate wavelengths where the transmission was 1%. [Chl] = 5 μg/ml; [DCMU] = 5 μM; temperature = 23° C.

TABLE 5.3

Cation Effects on the Degree of Polarization of Fluorescence for Different Conditions of Excitation and Observation

Treatment	Degree of Polarization of Fluorescence (%)					
	λexc. (nm):	480±16.5	480±16.5	480±5	435±5	480, Hg	436, Hg
	λobs. (nm):	F > 650	F > 694	← F686 →			
Salt-depleted		5.6	5.0	-	-	4.1	2.4
+ 10 mM NaCl		5.6	6.0	3.8	3.3	4.1	2.2
+ 10 mM NaCl + 10 mM $MgCl_2$		5.3	3.3	3.4	1.5	2.9	1.6

λexc. = wavelength of excitation; λobs. = wavelength of observation. The uncertainty values for the excitation wavelengths denote the half band-pass of the excitation monochromator. Hg denotes excitation with a mercury lamp at the respective emission line. [Chl] = 5 μg/ml; [DCMU] = 5 μM; temperature = 22-25° C.

this observation is not clear. On the other hand, it appears that the Mg^{2+} effect may sometimes be very prominent when excitation is with blue wavelengths. The prospects for optimization with blue excitation were not explored for three reasons: (1) The signal/noise ratio is lower with the blue excitation due to lower intensity of excitation in our instrument (tungsten or quartz iodine lamp). (2) The interpretation of chlorophyll a fluorescence polarization using Soret band excitation is difficult, if not controversial, even for pure chlorophyll in solution (cf. Weiss, 1972). (3) For the thylakoid system where a number of chromophores (e.g., Chl a, Chl b, and carotenoids) absorb blue light, serious difficulties are anticipated for the estimation of the relative contributions from these chromophores, a step necessary for energy transfer studies. Thus it appears that with our present understanding of the thylakoid system, and with the inherent difficulties mentioned above, definitive conclusions cannot readily be made on the pathway of energy transfer if blue excitation is used, and was thus avoided here in further measurements.

5.3.4 Reproducibility

The degree of polarization of fluorescence shows excellent reproducibility for different aliquots of the same thylakoid preparation. For thylakoids from different chloroplast isolations, however, slight variations in the degree of polarization of fluorescence are observed. The reproducibility of the cation effects is verified by measuring the changes in the degree of polarization of fluorescence on numerous

preparations of thylakoids. In these experiments the addition of cations is to the same suspension of thylakoids in any individual series. Typical results from thylakoids from three consecutive chloroplast preparations given in Table 5.4 show that the cation effects are readily reproducible.

5.3.5 Concentration Dependence

The Na^+-induced increase and the Mg^{2+}-induced decrease in the degree of polarization of the total fluorescence (RG 665 filter, F > 650 nm) saturate when the cation concentrations reach 4-6 mM (Table 5.5). Note also that when Mg^{2+} is added to thylakoids containing 10 mM Na^+, the fluorescence polarization assumes the value of the sample with Mg^{2+}. The converse, however, does not hold, demonstrating that when equal concentrations of Na^+ and Mg^{2+} are added the Mg^{2+} effect dominates.

5.3.6 Temperature Dependence

The degree of polarization of fluorescence from thylakoids under a particular cationic condition (salt-depleted, Na^+, or $Na^+ + Mg^{2+}$) is relatively constant between 0° and 25° C (see also Weber, 1960) showing that the mono- and di-valent cation effects are temperature independent over the range 0-25° C (Table 5.6).

5.3.7 O and P Level Dependence

The cation effects on the fluorescence polarization occur at both the O (initial) and the P (maximum) levels of the transient of fluorescence at 686 nm (Table 5.7).

TABLE 5.4

Cation Effects on the Polarization of Total Fluorescence in Three Consecutive Thylakoid Preparations

	$\underline{P}$(F > 650 nm), %		
Treatment	Sample 1	Sample 2	Sample 3
Salt-depleted	2.7 ± 0.16	2.8 ± 0.12	2.2 ± 0.11
+ 3 mM NaCl	3.0 ± 0.15	3.0 ± 0.17	2.6 ± 0.17
+ 3 mM NaCl + 3 mM $MgCl_2$	2.7 ± 0.15	2.9 ± 0.14	2.2 ± 0.19

Fluorescence excitation at 600 ± 8.3 nm, and detected through a Schott RG 665 glass filter. Lettuce thylakoids suspended in the usual sucrose medium, pH 7.5; [Chl] = 5 μg/ml; temperature = 22° C.

TABLE 5.5

Concentration Dependence of the Cation Effects on The Polarization of Fluorescence

$[Na^+]$, mM	$[Mg^{2+}]$, mM	P(F>694 nm), %
0	0	3.0
2	0	3.7
4	0	3.4
6	0	3.5
8	0	3.4
10	0	3.4
10	5	2.7
10	10	2.4
0	0	3.0
0	2	2.2
0	4	2.5
0	6	2.3
0	8	2.3
0	10	2.2
5	10	1.9
10	10	2.0

Excitation was at 600 ± 8.3 nm, and fluorescence was detected through a Schott RG 10 glass filter. Thylakoids from pea chloroplasts were suspended in the usual medium at pH 7.6; [Chl] = 5 µg/ml; [DCMU] = 5 µM; temperature = 23° C.

TABLE 5.6

Temperature dependence of the Cation Effects on the Intensity and Polarization of Fluorescence at 686 nm

	Salt-depleted		10 mM NaCl		10 mM NaCl + 10 mM $MgCl_2$	
Temperature, °C	F686	P	F686	P	F686	P
1.3	57	3.0	39	3.2	100	2.2
4.3	57	3.0	39	3.2	98	2.2
9.5	56	3.0	38	3.2	95	2.2
14.5	53	3.1	37	3.2	91	2.2
19.4	49	3.2	35	3.4	87	2.3
25.0	45	3.2	33	3.5	82	2.3

Fluorescence excited with light at 633 ± 6.6 nm, and detected through interference filter at 686 nm (FWHM, 6.4). Samples were pea thylakoids suspended in the usual medium, pH 7.6. [Chl] = 5 µg/ml; [DCMU] = 5 µM. Temperature was regulated to better than ± 0.5° C. P denotes the degree of polarization, and F the intensity of the fluorescence.

TABLE 5.7

Cation Effects on the Degree of Polarization at the Initial (O) and Maximum (P) Levels of Fluorescence Transient at 686 nm

	P(F686), %	
Sample	O Level	P Level
Salt-depleted	3.9	3.7
+ 5 mM NaCl	4.8	4.1
+ 5 mM NaCl + 5 mM $MgCl_2$	3.6	3.1

Fluorescence excited at 600 ± 6.6 nm, and detected with 686 nm interference filter (FWHM, 6.4 nm) plus Schott RG 665 filter. Samples consisted of pea thylakoids suspended in the usual medium at pH 7.2; temperature = 23° C.

5.3.8 Emission Wavelength Dependence

Studies were made at four different emission wavelengths isolated by interference filters. The rationale for the choice of the measuring wavelengths at 686 (F686), 712 (F712), 730 (F730) and 762 nm (F762) was as follows. The emission spectrum of chlorophyll *a* fluorescence in thylakoids at room temperature shows a major band at ~685 nm with a minor band at ~730 nm (vibrational satellite of the 685 nm band). Although most of this fluorescence originates from PS II (see Govindjee *et al.*, 1973), the fraction of PS II to PS I fluorescence attains a minimum in the 710-720 nm region (Lavorel, 1962; Vredenberg and Duysens, 1965; Briantais, 1969). From his studies on separated pigment systems, Briantais (1969) has shown that fluorescence from isolated PS I particles, at room temperature, has a broad peak in the 710-720 nm region. Thus, although F685, F730 and F760 represent mainly PS II, F712 would have a higher proportion of PS I fluorescence than at other wavelengths. This is further substantiated here by two additional observations:

(1) The degree of polarization of chlorophyll *a* fluorescence at 712 nm is higher than that at 686 or 730 nm (Table 5.8), and it is known that the degree of polarization of fluorescence from isolated PS I particles is higher than in PS II particles (Cederstrand and Govindjee, 1966; Gasanov and Govindjee, 1974).

(2) We have also found that the Mg^{2+} enhancement of fluorescence intensity varies depending on the emission wavelength. This variation, given as the ratio of the emission in the presence of Mg^{2+} to that in

TABLE 5.8

Effects of Na^+ and Mg^{2+} on the Degree of Polarization of Chlorophyll *a* Fluorescence in the Presence of 5 μM DCMU

	Degree of Polarization (%)		
Treatment	P(F686)	P(F712)	P(F730)
Salt-depleted	2.2 ± 0.1	4.3 ± 0.3	2.9 ± 0.1
+5 mM NaCl	3.1 ± 0.3	2.3 ± 0.3	4.2 ± 0.2
+5 mM NaCl +5 mM $MgCl_2$	2.0 ± 0.2	4.2 ± 0.3	3.8 ± 0.2

Fluorescence was excited at 600 nm (band-pass, 16.5 nm) with vertically polarized light and detected through interference filters--at 686 nm (FWHM, 6.4 nm), at 712 nm (FWHM, 5.2 nm), and at 730 nm (FWHM, 8.4 nm)--with an EMI 9558 B (S-20 response) photomultiplier. Thylakoids were suspended in 100 mM sucrose containing 0.4 mM Tris-HCl at pH 7.6 and a chlorophyll concentration of ~5 μg/ml.

The results are the average of three experiments; the effects were further confirmed in two other measurements. The errors denote ± one standard deviation.

its absence at the corresponding wavelength, shows a minimum in the region of 710-720 nm over the pH range 5-9--this is shown later in Chapter 6, see Fig. 6.3.

Table 5.8 shows the effects of mono- and di-valent cations on the degree of polarization, P, of Chl a fluorescence at room temperature (excitation at 600 ± 8.3 nm) as a function of the emission wavelengths at 686 nm (F686; PS II), at 712 (F712; PS I), and at 730 (F730; PS II). The degree of polarization of F686, P(F686), change from 2.2 ± 0.1% to 3.1 ± 0.3% to 2.0 ± 0.2% from salt-depleted to 5 mM NaCl to 5 mM NaCl + 5 mM $MgCl_2$ condition. This shows that the addition of 5 mM Na^+ increased P(F686) by 26-56% and the subsequent addition of 5 mM Mg^{2+} reversed this increase. Similar effects were observed for P(F730). For F712, however, the addition of 5 mM Na^+ to salt-depleted thylakoids changed P from 4.3 ± 0.3% to 2.3 ± 0.3% (a decrease of 39-51%), which was then reversed by the addition of 5 mM Mg^{2+}. It is noted that the results in Table 5.8 for P(F712) show some of the largest effects we have measured. Very often cations show no apparent effects on P(F712), even when those at 686 and 730 nm are clearly observed. This situation is relatively simple to rationalize, since only two photosystems contribute to the fluorescence at any wavelength of emission and the one mainly contributing to the fluorescence at 686 and 730 nm is PS II. Since the cation effects on the fluorescence polarization at 686 and 730 nm are always present, the observation of no change in P(F712) implies that the effects on the degree of polarization of fluorescence from PS I are opposite to those from PS II.

The above results suggest that Na^+ induces an increase in P from PS II and a decrease in that from PS I. Further addition of Mg^{2+} causes an antagonistic effect, i.e., a complete reversal of these effects.

In another set of experiments, the degree of polarization of F762, P(F762), was found to decrease with Mg^{2+} addition to the Na^+ samples at all excitation wavelengths (λ_{ex}) in the range $600 \leq \lambda_{ex} < 700$ nm. (The observation that the cation effects on P(F762) are similar to P(F686) and P(F730) suggests that F762 also reflects the fluorescence changes of Chl a_{II}).

5.3.9 Excitation Wavelength Dependence

The degree of polarization as a function of excitation wavelength for the Na^+ and the $Na^+ + Mg^{2+}$ samples are given in Fig. 5.3. The excitation spectrum between 635 and 700 nm for the change in P(F762) of the $Na^+ + Mg^{2+}$ sample compared to the Na^+ sample (Fig. 5.4) shows negative bands at ~650 (Chl b), ~675 (Chl a), and 685 nm (Chl a). The excitation spectra for the Mg^{2+}-induced changes in F730 and F762 (Fig. 5.5) show a general enhancement of fluorescence yield by Mg^{2+} addition, with a peak at ~650 nm and a shoulder at ~675 nm. The difference excitation spectra between the thylakoid suspension with Mg^{2+} and that without Mg^{2+} for F730 and F762 are identical (Fig. 5.6) and show the two bands clearly at 650 and 675 nm. These results on the Mg^{2+} effects in Figs. 5.3 to 5.6 are best explained in terms of (a) an increase in energy transfer from Chl LH to Chl a_{II}, and (b) a decrease in transfer from PS II to PS I (see Discussion).

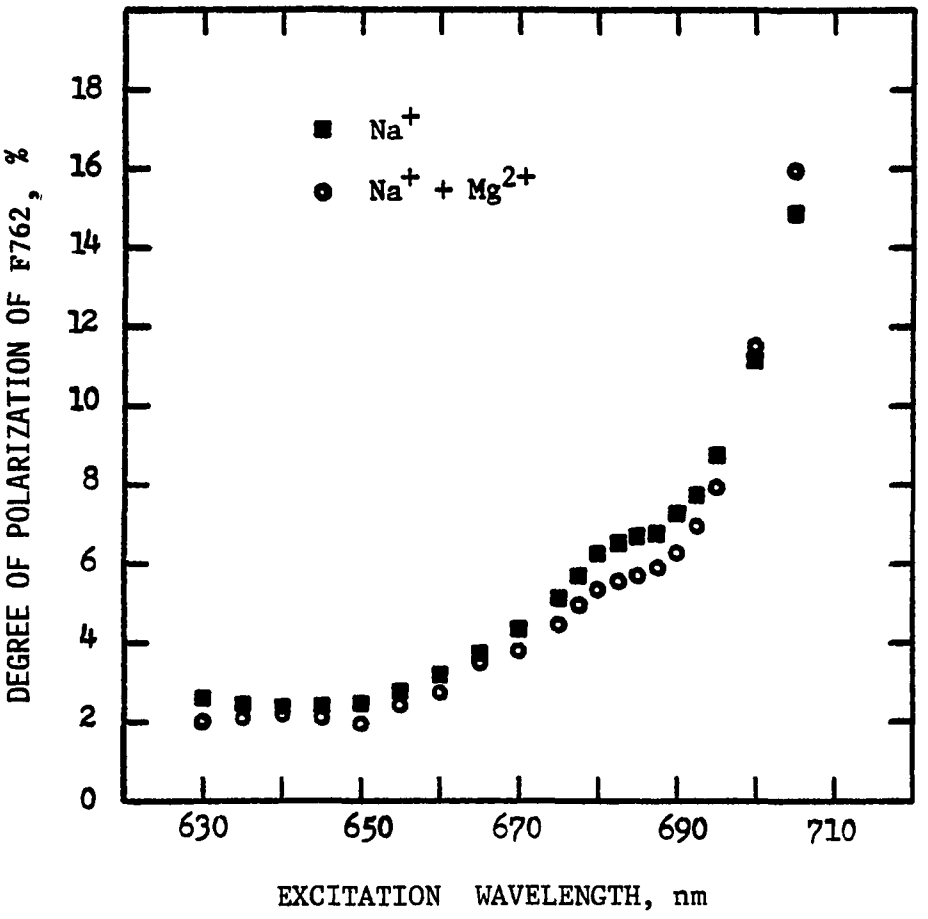

Figure 5.3 Degree of Polarization of Fluorescence at 762 nm (FWHM, 11.3 nm) versus Excitation Wavelength (band-pass, 5 nm). Sample details as given in the legend of Table 6.1.

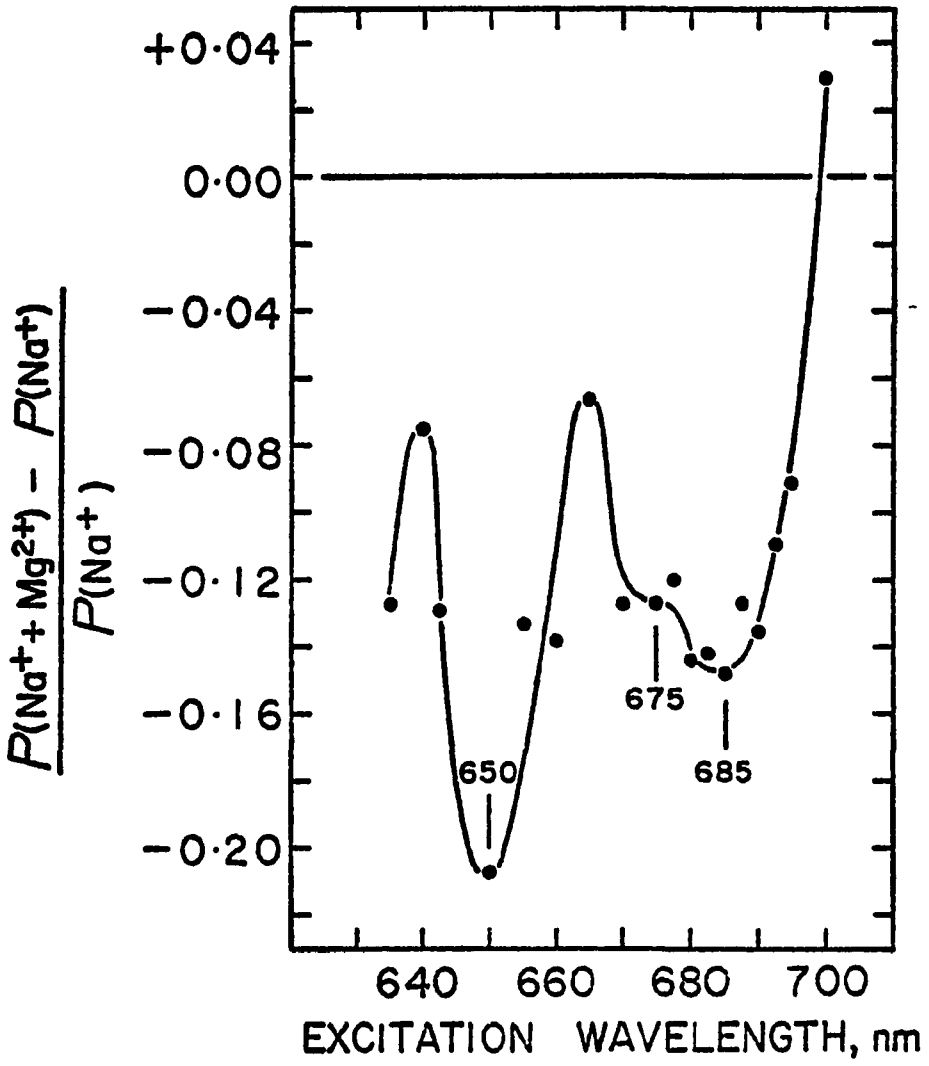

Figure 5.4 Excitation Spectrum of the Relative Difference in the Degree of Polarization of F762 between the $Na^+ + Mg^{2+}$ Sample and the Na^+ Sample Expressed as $[\underline{P}(Na^+ + Mg^{2+}) - \underline{P}(Na^+)]/\underline{P}(Na^+)$. The excitation band-pass was 5 nm. The fluorescence was detected through a combination of a Schott RG 10 glass filter and an interference filter at 762 nm (FWHM, 11.3 nm). Sample details are as given in the legend of Table 5.8.

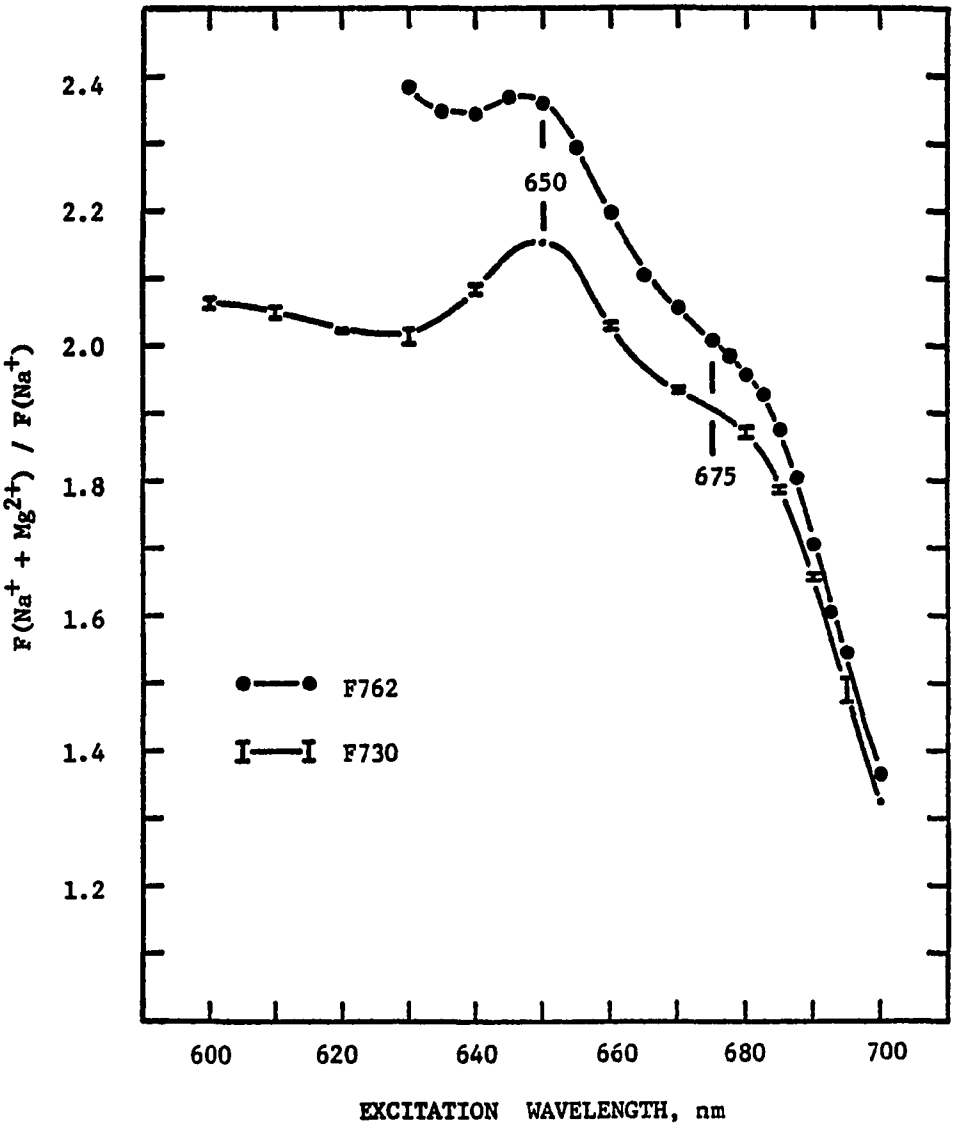

Figure 5.5 Excitation Spectra of Mg^{2+} Enhancement of Fluorescence at 730 and 762 nm. Experimental details as given in the legend of Fig. 5.4.

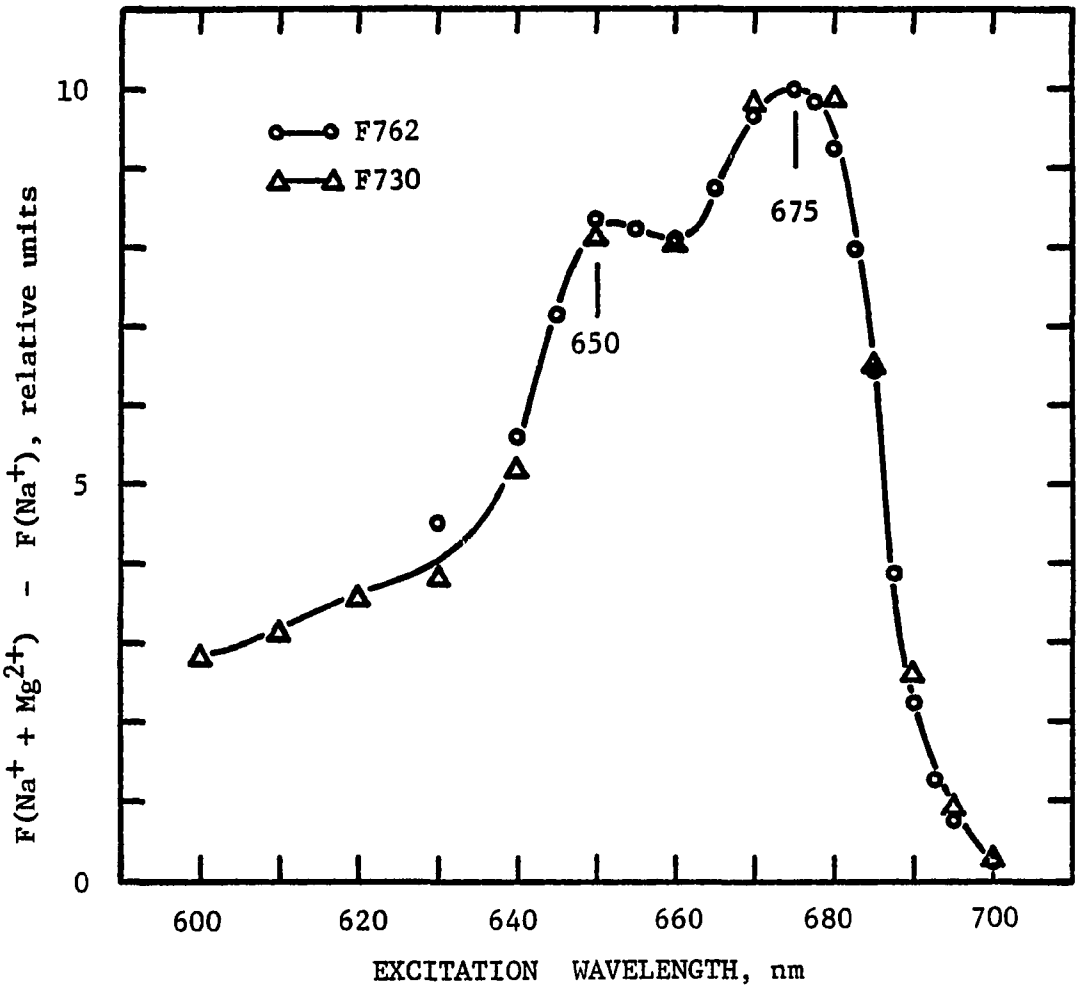

Figure 5.6 Difference Excitation Spectrum, between $Na^+ + Mg^{2+}$ and Na^+ Samples, for Fluorescence at 730 and 762 nm, Normalized at 675 nm. Experimental details as given in the legend of Fig. 5.4.

Observations were also made on F730 with and without 43% (v/v) glycerol to reduce sample turbidity. Results very similar to those in Figs. 5.3 to 5.5 were obtained, although the Mg^{2+} effect beyond 690 nm on P(F762) without glycerol and P(F730) with and without glycerol were indeed identical to those in Figs. 5.4 and 5.5 and are taken as confirmation that the observed changes are not the result of differences in sample scattering.

5.4 Discussion

5.4.1 General Comments

The DCMU-induced increase in yield and depolarization of Chl *a* fluorescence and the decline of the depolarization effect with increasing wavelengths of excitation (finally disappearing at ~680 nm) strongly support the concept that by keeping the PS II reaction centers closed, DCMU enhances the extent of energy migration among the less mutually aligned chlorophyll molecules absorbing at wavelengths <680 nm (Mar and Govindjee, 1972; Whitmarsh and Levine, 1974; Becker *et al.*, 1976). DNB, on the other hand, is an excellent quencher of chlorophyll fluorescence *in vitro* (Livingston and Ke, 1950) and *in vivo* (Teale, 1960; Amesz and Fork, 1967; Lavorel and Joliot, 1972; Etienne *et al.*, 1974). Its influence in decreasing the extent of energy transfer is based on the kinetics of the photochemical O to P phase of the fluorescence transient (Lavorel and Joliot, 1972). The direct relation between fluorescence quenching and depolarization (Fig. 5.2) and their linear dependence on the concentration of DNB (Fig. 5.1; cf. Teale, 1960)

demonstrate the inverse relation between the fluorescence yield and degree of polarization in the case of fluorescence quenching. The above results with DCMU and DNB empirically support the occurrence in thylakoids of the inverse relation between the extent of energy transfer and the degree of polarization of the fluorescence.

To facilitate understanding of the results, excitation was kept only to the red region of the spectrum, as carotenoids further complicate interpretations in the blue region. The reproducibility of the cation effects on the polarization of fluorescence at 686 and 730 nm is excellent. The changes at 712 nm may frequently be difficult to recognize because of the narrow wavelength region where PS II/PS I emission has a relative minimum at 298 K. The cation-effects on fluorescence polarization saturate at 4-6 mM. No significant temperature dependence between 0 and 25° C, nor any dependence on whether the fluorescence is observed at the O or P level.

The present chapter provides important experimental data for the conclusion that cations cause changes in the excitation energy transfer in the photosynthetic system at physiological temperatures. This is shown by an increase and a decrease in the polarization of PS II and PS I fluorescence, respectively, by monovalent cations, and the reversal of these effects by further addition of divalent cations (Table 5.8). Moreover, the increases in depolarization by divalent cations have maxima at ~650 (Chl *b*), ~675 (Chl *a*), and 685 nm (Chl *a*) for fluorescence measured at 762 nm (Fig. 5.4), as well as at 730 nm. At the same time, the fluorescence enhancement shows peaks at ~650 and

~675 nm (Fig. 5.5). These data strongly suggest an increase in net energy transfer from light-harvesting chlorophyll protein (LHC) (Chl *b* and Chl *a* 670) to Chl a_{II}. The presence of another band at 685 nm in Fig. 5.4, suggests that there is an additional effect on Chl *a* 685; this effect may be due to a change in the orientation of key chlorophyll *a* molecules that control PS II to PS I energy transfer, a concept suggested earlier by Seely (1973) (see also VanderMeulen and Govindjee, 1974).

5.4.2 Changes in Interunit Excitation Energy Transfer

In order to appreciate the above conclusions and to know their limitations we remind the reader of several well-known and some less-established basic concepts: (1) there are two pigment systems PS I and PS II (cf. Williams, 1977); all chlorophylls can be divided into, at least, three functional forms of chlorophyll-protein complexes; LHC, Chl a_{II}, and Chl a_{I} (see Boardman *et al.*, 1978); (2) excitation energy can be shared between several PS II units (Joliot *et al.*, 1973), where a unit is understood here as a group of chlorophyll molecules serving a reaction center (cf. Duysens, 1964); (3) intra-unit transfer is complete within an interval that is short compared to the fluorescence lifetime (cf. Michel-Villaz, 1976), and thus increases in intra-unit energy transfer do not significantly affect the degree of polarization of fluorescence; (4) inter-unit energy transfer decreases the fluorescence polarization (Michel-Villaz, 1976). Within this framework of ideas, our observation that Na^+ caused a decrease in the polarization

of PS I fluorescence, and an increase in the polarization of PS II fluorescence (Table 5.8) suggests that the addition of Na^+ causes an increase in inter-unit transfer from PS II to PS I, and a decrease in inter-unit transfer among PS II units (also see Moya et al., 1977). Subsequent addition of Mg^{2+} reverses these changes.

5.4.3 Is Change in Orientation of Chl a 685 Cause of Quanta Redistribution?

The concept that a change in the orientation of a few key chlorophyll a molecules can significantly alter the rate of energy transfer from PS II to PS I was suggested by Seely (1973). Recently, Biggins and Svejkovsky (1978) have observed Mg^{2+}-induced changes in linear dichroism spectra of magnetically-oriented chloroplast membranes. They have suggested that Mg^{2+} causes a species of chromophores (Chl a 690) to reorient closer into the plane of the thylakoid membrane. Since we observe (Fig. 5.4) an increase in the depolarization of fluorescence with 685 nm excitation upon Mg^{2+} addition, this could be due to a decrease in the mutual order in the Chl a 685 chromophore group(s). Whether these two effects are due to Mg^{2+} effects on the same chromophores cannot be decided yet, although it is tempting to speculate that the 690 nm band in the experiments of Biggins and Svejkovsky may have been slightly shifted to longer wavelengths by selective scattering (see Latimer, 1959). Our interpretation of the fluorescence polarization decrease is not inconsistent with that of Biggins and Svejkovsky (1978) for linear dichroism

increase in magnetically-oriented suspensions, as one can envisage a simultaneous reorientation of a long-wavelength species of chlorophyll a molecules closer into the plane of the membrane and a decrease in their mutual alignment. This explanation is also consistent with the picture presented in the previous paragraph because Becker et al. (1976) have suggested that chlorophyll a molecules absorbing at ≥680 nm have a high mutual order, so that increased energy transfer between these molecules have little consequence on the fluorescence polarization. Thus, changes in polarization of fluorescence with excitation at 685 nm may safely be taken as a reduction of mutual orientation of Chl a 685. Finally, since linear dichroism and fluorescence polarization spectral studies (cf. Garab and Breton, 1976) suggest that the shorter wavelength forms of chlorophyll a (absorbing ≤670 nm) are less aligned with respect to the membrane plane, i.e., sustaining a greater angle with the plane, a reorientation of Chl a 685 closer to the membrane plane would decrease the orientation factor (Förster, 1965) between Chl a 670 (in PS II) and Chl a 685 (in PS I) decreasing energy transfer from PS II to PS I.

5.4.4 Change in Coupling of Chlorophyll a/b Complex with Chl a_{II}

On the other hand, changes in polarization of fluorescence excited at shorter wavelengths (in Chl a 670, Chl b 650, etc.) have been suggested to be due to changes in excitation energy transfer between chlorophyll species with low mutual order (Michel-Villaz, 1976; Becker et al., 1976). Thus, the negative peaks at ~650 nm (due to Chl b) and

at ~675 nm (due to Chl *a* 670) in the Mg^{2+}-induced changes in polarization of Chl a_{II} fluorescence (Fig. 5.4) and positive peaks at ~650 nm and ~675 nm in the Mg^{2+}-induced increase in relative fluorescence yield (Figs. 5.5 and 5.6) are interpreted to be due to an enhancement of energy transfer from these complexes (present in LHC) to fluorescent Chl a_{II}. It is proposed that this is the process which increases the initial distribution of quanta from LHC to PS II. The observation that the relative enhancement of fluorescence of Chl *b* is small (Fig. 5.5; ~6% for F730 is consistent with previous findings (Butler and Kitajima, 1975a; Moya *et al.*, 1977; Wong, *et al.*, 1978, 1979--Chapters 3 and 4) that the Mg^{2+}-induced variations in the sensitization of PS II fluorescence is ≤20%. These changes are, perhaps, manifestations of the proposed Mg^{2+}-induced increase in the coupling of LHC with Chl a_{II} (Arntzen and Ditto, 1976; see also Butler and Strasser, 1978; Paillotin, 1978).

The observation of peaks at ~650 and ~675 nm in Figs. 5.4 to 5.6 is the first unambiguous demonstration of a Mg^{2+}-induced increase in Chl LH→Chl a_{II} transfer in normal thylakoids at physiological temperatures. A brief discussion of the two previous studies is given below. Vernotte *et al.* (1973) showed that Mg^{2+} causes no change in the action spectrum of PS II reactions while it inhibits PS I reactions with a spectrum having peaks at ~650 and ~670 nm. These results may be interpreted equally well by a Mg^{2+}-induced inhibition of initial quanta distribution from LHC to Chl a_I, as by a Mg^{2+}-induced inhibition of redistribution of quanta from PS II (initially transferred there from

LHC) to PS I. Loos (1976), on the other hand, showed changes in the action spectra of the variable yield fluorescence and oxygen evolution (PS II) and O_2 uptake (Mehler reaction, PS I) at 480 nm, but could not unequivocally distinguish between the involvement of Chl *b* and carotenoids. Interpretation of the results in Figs. 5.4 to 5.6 is definitive for two reasons: (1) By limiting the actinic light to wavelengths longer than 600 nm (exciting only the chlorophylls), complications of interpretation from the possible involvement of carotenoids in excitation energy transfer are avoided, and (2) by the simultaneous evaluation of the fluorescence intensity and polarization results, the possibility for ambiguities of interpretation of the type encountered with the data of Vernotte *et al.* (1973) is eliminated. The importance of simultaneous evaluation of the fluorescence data must not be overlooked. For instance, with only the fluorescence depolarization results, even if it could be independently established that Mg^{2+} induces an increase in the extent of energy transfer, it would not be possible to conclude whether the change results from an increase in the initial partition of quanta to PS II, or an inhibition of their subsequent redistribution from PS II to PS I. However, if the inhibition of quanta redistribution were the only cause of the fluorescence polarization changes, the same relative enhancement of fluorescence intensity by Mg^{2+} would be observed whether Chl a_{II} or Chl LH were excited. The observation in Fig. 5.6 that the action spectrum for the Mg^{2+} enhancement of Chl a_{II} fluorescence intensity contains relative maxima at ~650 and ~675 nm implies quite conclusively that variations in the initial

partition of energy are the result of changes in the energy coupling between Chl LH and Chl $\underline{a}_{II}$.

5.5 Concluding Remarks

Since the discovery by Homann (1969) and Murata (1969) that divalent cations increase the relative Chl *a* fluorescence yield even in the presence of the electron transfer inhibitor DCMU, various investigators (see reviews by Barber, 1976 and Williams, 1977) have attempted to find the molecular mechanism by which cations affect the photoprocesses in thylakoids. Arntzen and coworkers (1978) have shown that LHC is necessary for the cation-induced regulation of the excitation energy transfer. Our results show that divalent cations affect Chl *b* and Chl *a* 675 (components in LHC) in such a way that there is increased energy transfer from these chromophores to Chl $\underline{a}_{II}$ (increased energy coupling). In addition, it is concluded that the mutual orientation of Chl *a* 685 is decreased by Mg^{2+} supporting Seely's hypothesis (1973) of how cations may regulate excitation transfer from PS II to PS I. Decreased energy transfer from PS II to PS I seems to be accompanied by an increase in inter-unit transfer among PS II units (cf. Moya *et al.*, 1977).

In summary, the results of this study are consistent with the following: (a) there is an antagonistic effect of low concentrations (3-5 mM) of mono- and di-valent cations on the excitation energy migration, Na^{+} causing less energy migration among PS II units and more transfer to PS I--both the effects being reversed by Mg^{2+}; and (b) divalent cations increase excitation energy transfer from both

chlorophyll *a* and chlorophyll *b* (contained in the light harvesting chlorophyll *a*/*b* protein complex, LHC) to chlorophyll *a* in PS II. Conclusion (a) supports Murata's hypothesis (1969) of cation regulation by excitation energy redistribution from PS II to PS I, and conclusion (b) may be taken to be in agreement with the concept of Arntzen and Ditto (1976) that divalent cations increase the coupling between LHC and chlorophyll *a* of PS II (Chl a_{II}).

CHAPTER 6

BULK pH AND THE CATION EFFECTS AT ROOM TEMPERATURE AND 77 K

6.1 Introduction

We shall now present the effects of varying the bulk H^+ ion concentration in the suspension medium on the excitation energy regulation in thylakoids. This investigation was undertaken for the following reasons. Firstly, the analyses in the preceeding chapters were made for thylakoids suspended in medium at neutral pH, and it is important to know the pH range for which these conclusions are valid. This is particularly significant in view of the fact that the pH of the intrathylakoid space (loculus) show large variations in pH in normal operation (see discussion in Barber, 1976). Secondly, among the most commonly cited evidence supporting the cation effects on excitation distribution and redistribution between the photosystems are the opposite changes in the measured electron transport rates of PS I and PS II partial reactions under light-limiting conditions. However, it was found (Bose, 1974) that at pH > 7.5 Mg^{2+} caused a decrease in the rate of $NADP^+$ reduction (as initially reported by Murata, 1969), but at pH < 7.5 Mg^{2+} caused an enhancement in this rate. Similar results have recently been reported by Rurainski and Mader (1978) for electron transport from $DCPIPH_2$ to $NADP^+$. Since it is known (see Harnischfeger and Shavit, 1974) that the binding of ferredoxin required for $NADP^+$ reduction is Mg^{2+} sensitive, it is essential to test whether these changes in $NADP^+$ reduction show a direct correlation to the rate of quanta input to PS I.

Finally, recent studies by Barber and coworkers (cf. Barber et al., 1977) have shown that the cation effects on chlorophyll fluorescence show a satisfactory correlation to the membrane surface charge density. The latter finding suggests that the cation effects should be pH sensitive, and a comprehensive study by parallel measurements of the pH dependences of the cation effects on various fluorescence and electron transport parameters in the two photosystems could provide further insight into the phenomenon.

6.2 Materials and Methods

Thylakoids were prepared as described in Chapter 2. The final suspension medium used in all experiments was 100 mM sucrose containing 2 mM Tris adjusted to an appropriate pH with HCl or HNO_3. Since the concentrated stock thylakoid membranes, suspended in 100 mM unbuffered sucrose, showed slightly acidic pH's, the final pH of each sample (pH individually measured) was 0.4-0.8 pH unit lower than the pH of the dilution medium. Other details were as given in the legends of the tables and figures or in Chapter 2.

6.3 Results

6.3.1 Cation Effects on Chlorophyll a Fluorescence

6.3.1.1 Cation Concentration Curves for Maximum Flourescence in DCMU-Treated Thylakoids

The optimum concentrations of cations to be used were determined from a sampling of the cation concentration dependence of the steady-state or maximum fluorescence (F_M) at 685 nm in salt-depleted thylakoids

treated with DCMU at pH's 6.2, 7.1, and 8.6. The results for Na^+ and Mg^{2+} are given in Fig. 6.1. With F_M for the salt-depleted sample in each case normalized to 1.0, the following general characteristics are noted:

(1) There are two phases in the cation (mono- and di-valent) concentration curves for F_M. For divalent cations, F_M increases rapidly for cation concentrations in the range 0-10 mM (attaining a maximum at 10 mM) and then declines slightly as the concentration increases from 10 to 100 mM. In the case of monovalent cations, however, F_M shows an "S"-shaped type dependence on concentration; at low concentrations (20-40 mM depending on pH), Na^+ induces a decrease in F_M, but, beyond ~40 mM, Na^+ causes an increase in F_M, saturating in the region of ~100 mM. At ~100 mM concentrations, both mono- and di-valent cations give approximately the same relative fluorescence yield.

(2) F_M in the presence of 10 mM Mg^{2+} is highest at pH 7.1, followed by pH 6.2 and then pH 8.6. Ten mM Na^+, on the other hand, induces a greater decrease (~14%) in F_M at pH 8.6 than pH 7.1; at pH 6.2 there is only a very slight change in F_M.

(3) The half-saturation concentration for the Mg^{2+}-induced increase in F_M shifts to lower values with increasing pH (Fig. 6-2).

6.3.1.2 pH Dependence of Cation Effects on Chlorophyll a Fluorescence at Room Temperature

EMISSION SPECTRA. The room temperature flourescence emission spectra for thylakoids suspended at pH 6.2 in media containing 10 mM Na^+ and 10 mM Na^+ + 10 mM Mg^{2+} are shown in Fig. 6.3. Corresponding

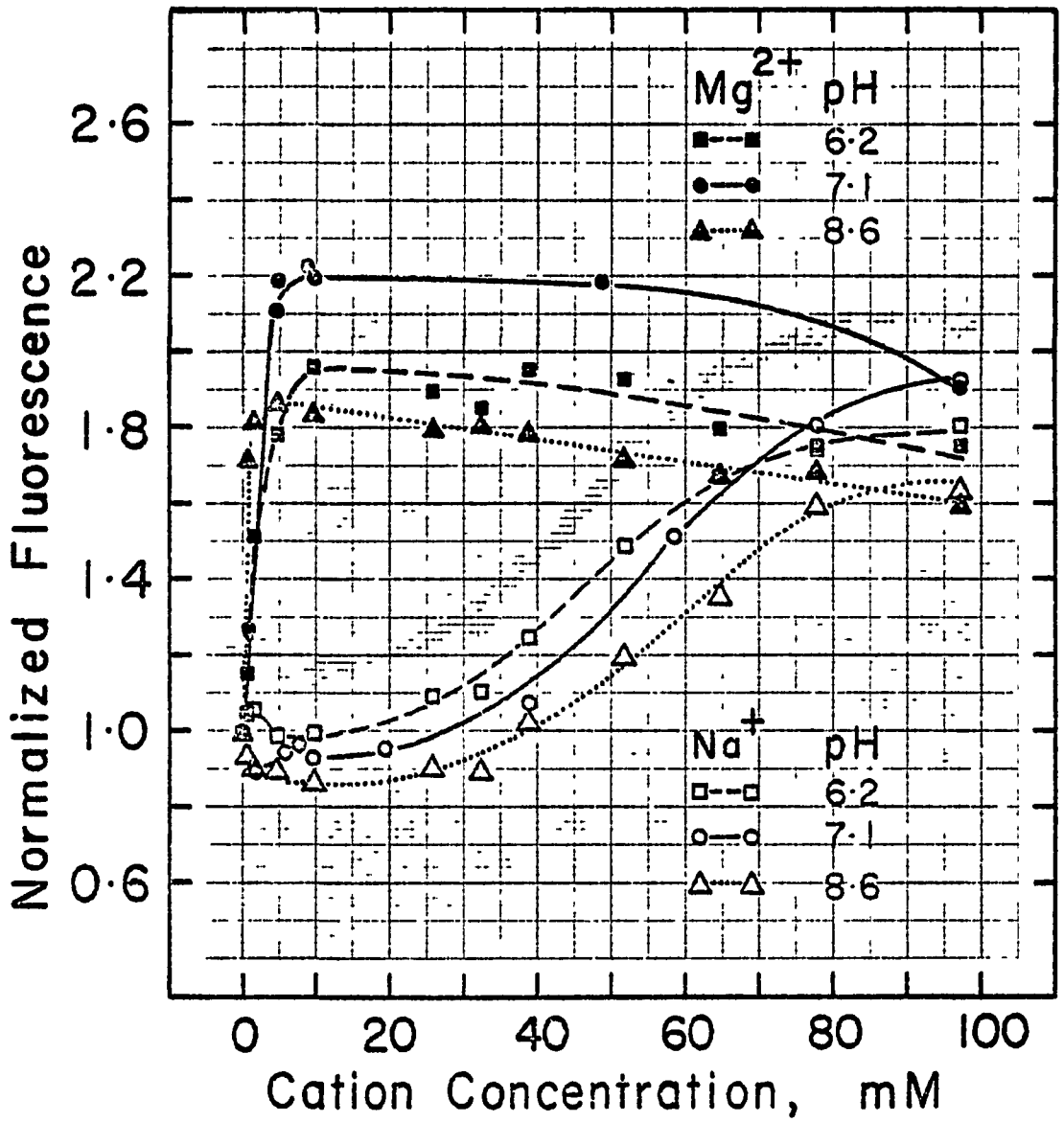

Figure 6.1 Fluorescence Intensity at 685 nm as Function of the Cation Concentration, at Different pH's. Thylakoids suspended in 100 mM sucrose + 2 mM Tris-HCl (appropriate pH); [Chl] = 5 µg/ml; [DCMU] = 3.3 µM; temperature = 23° C. Excitation at 636 nm (FWHM, 8 nm). Experimental details as described in Chapter 2.

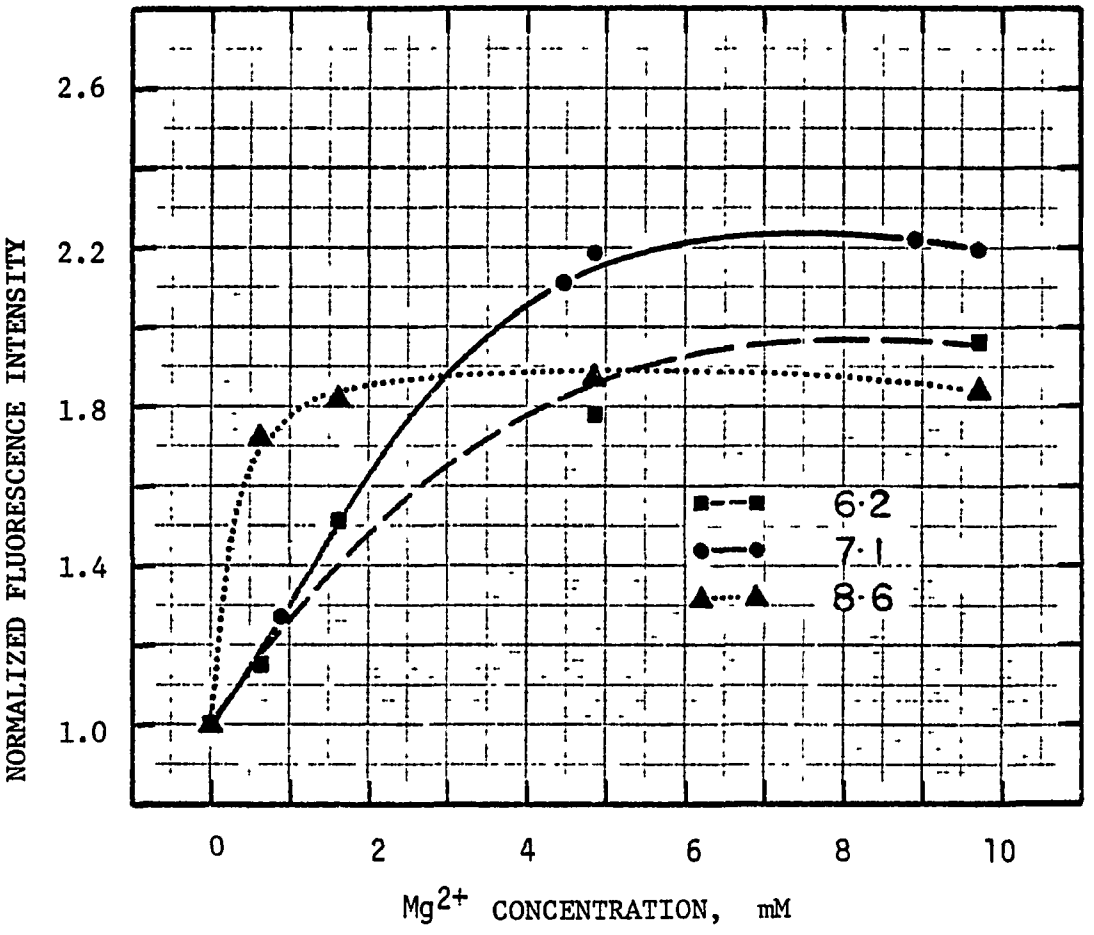

Figure 6.2 Fluorescence Intensity at 685 nm as Function of Mg^{2+} Concentration. The same data points as in Fig. 6.1 for $[Mg^{2+}]$ between 0 and 10 mM are replotted on an expanded scale for concentration.

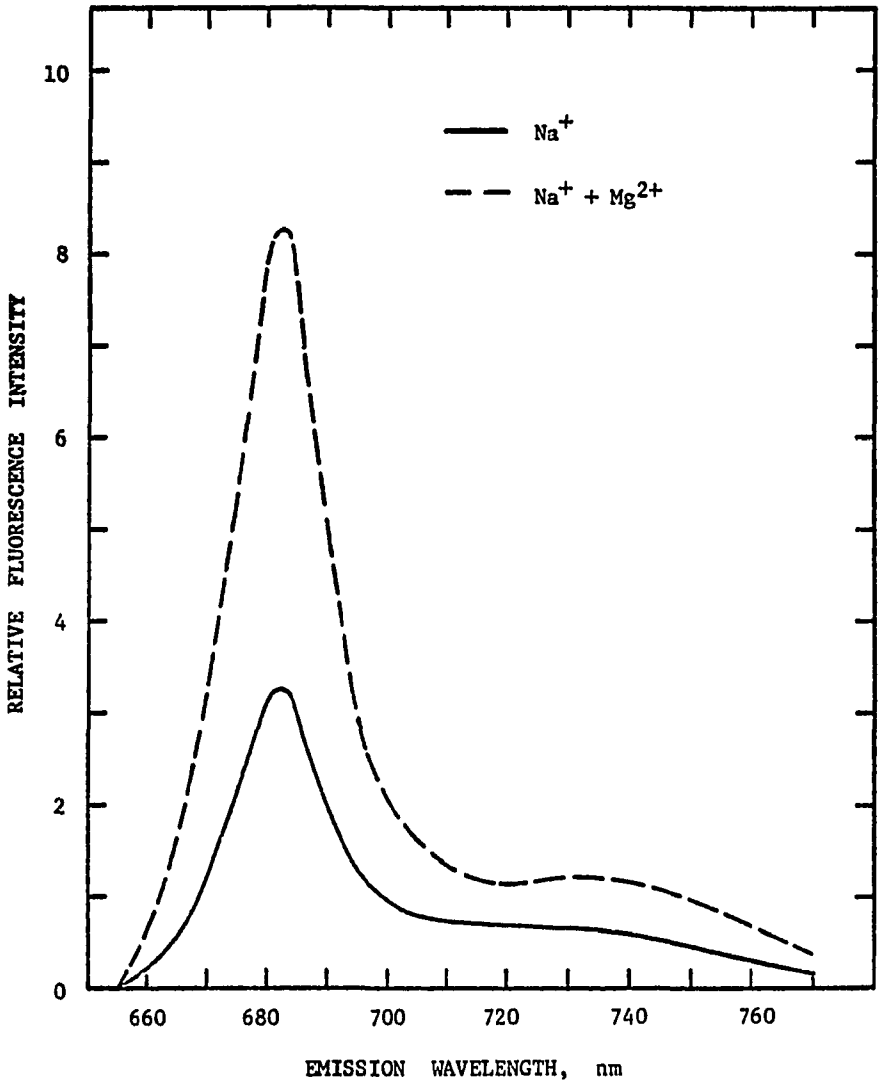

Figure 6.3 296 K Fluorescence Emission Spectra of the Na^+ and Na^+ + Mg^{2+} Samples at pH 9. Excitation was at 636 nm (FWHM, 8 nm) and Fluorescence was detected through a Schott RG 665 glass filter and a monochromator (band-pass, 6.6 nm). All spectra were corrected for transmission characteristics of filter and monochromator, photomultiplier sensitivity, and stray light as described in Chapter 2. [Chl] = 5 µg/ml; [Na^+] = Mg^{2+}] = ~10 mM; [DCMU] = 3.3 µM.

spectra at pH's 5.3, 7.1, and 8.6 are similar to those at pH 6.2, although the relative enhancement by Mg^{2+} differ (data given below). In the pH range from 5.3 to 8.6 the main emission peak is at ~685 nm with the vibronic band at ~730 nm. The Mg^{2+}-induced increase in fluorescence shows a strong dependence on emission wavelength. The ratios of the emission spectra of samples containing Mg^{2+} to the corresponding spectra without Mg^{2+} at various pH's are plotted in Fig. 6.4. It seems that in all the cases (except at pH 5.3 where there is only a slight change) the ratio spectrum shows a large Mg^{2+}-induced enhancement of fluorescence between 665 and ~690nm, but that this enhancement significantly declines beyond ~690 nm to a minimum at 710-720 nm, followed by a significant rise and perhaps a final decline beyond 750 nm.

MAXIMUM STEADY-STATE FLUORESCENCE. The above results (Fig. 6.1 to 6.4) confirm that the optimum conditions for the study of cation effects on chlorophyll a fluorescence in the pH range 6-9 is to observe the emission at ~685 nm in the presence of 10 mM cations. With these conditions ascertained, the maximum steady-state fluorescence (F_M) of thylakoids suspended in cation free, Na^+, and $Na^+ + Mg^{2+}$ media in the presence of DCMU were measured. The averaged results for thylakoids from three different chloroplast isolations are given in Fig. 6.5. The pH profiles of F_M for the three cationic conditions of the suspension medium are clearly different. For sucrose-washed thylakoids in cation-free medium, F_M shows an increase from pH 5.0 to a peak at pH ~6.3, decreasing thereon to a low value at pH ~7.7 (open circles). With

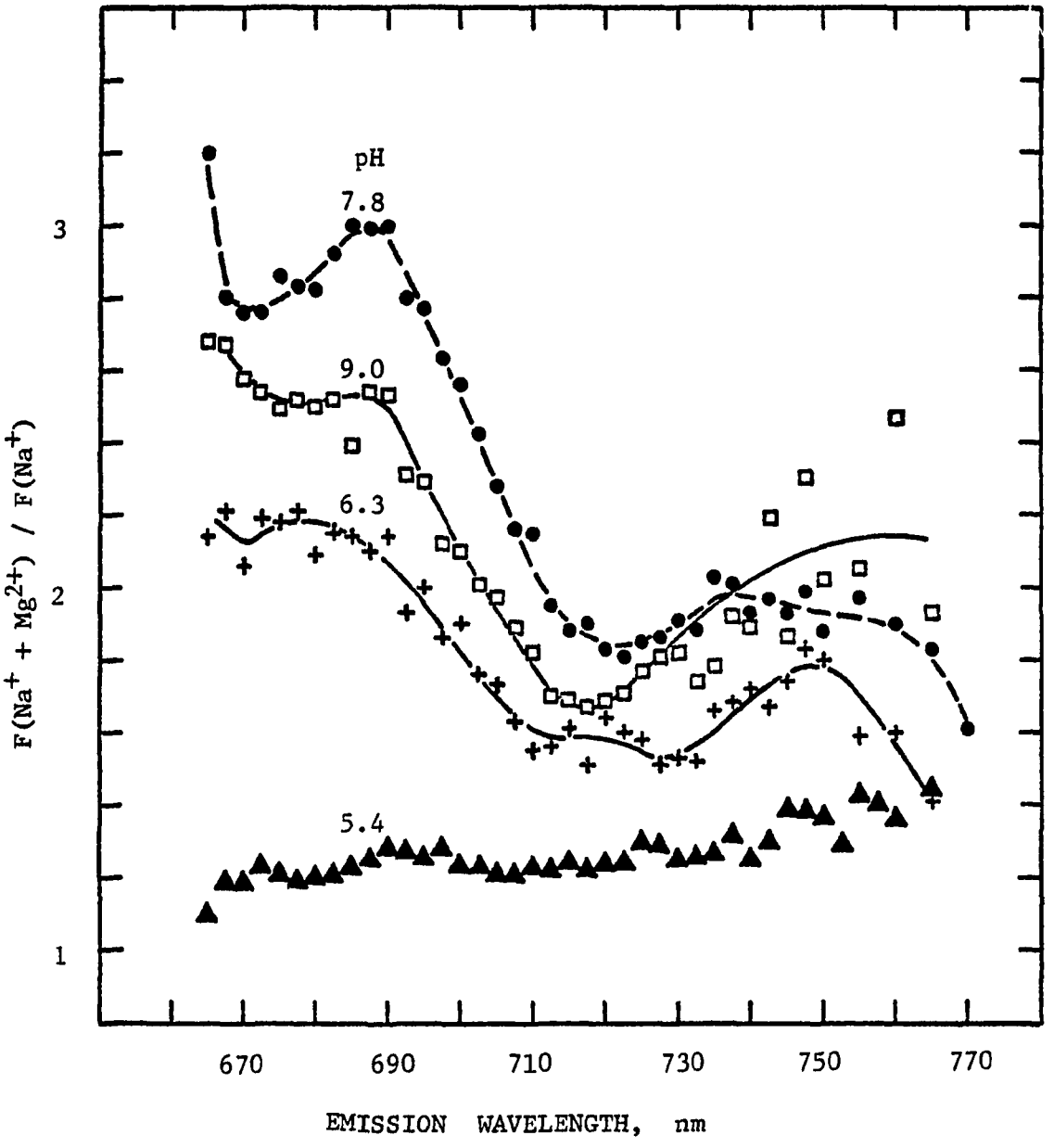

Figure 6.4 Fluorescence Intensity Ratio between the $Na^+ + Mg^{2+}$ and the Na^+ Sample as Function of Emission Wavelength, at Different pH's. Similar conditions to those in Fig. 6.1 were used.

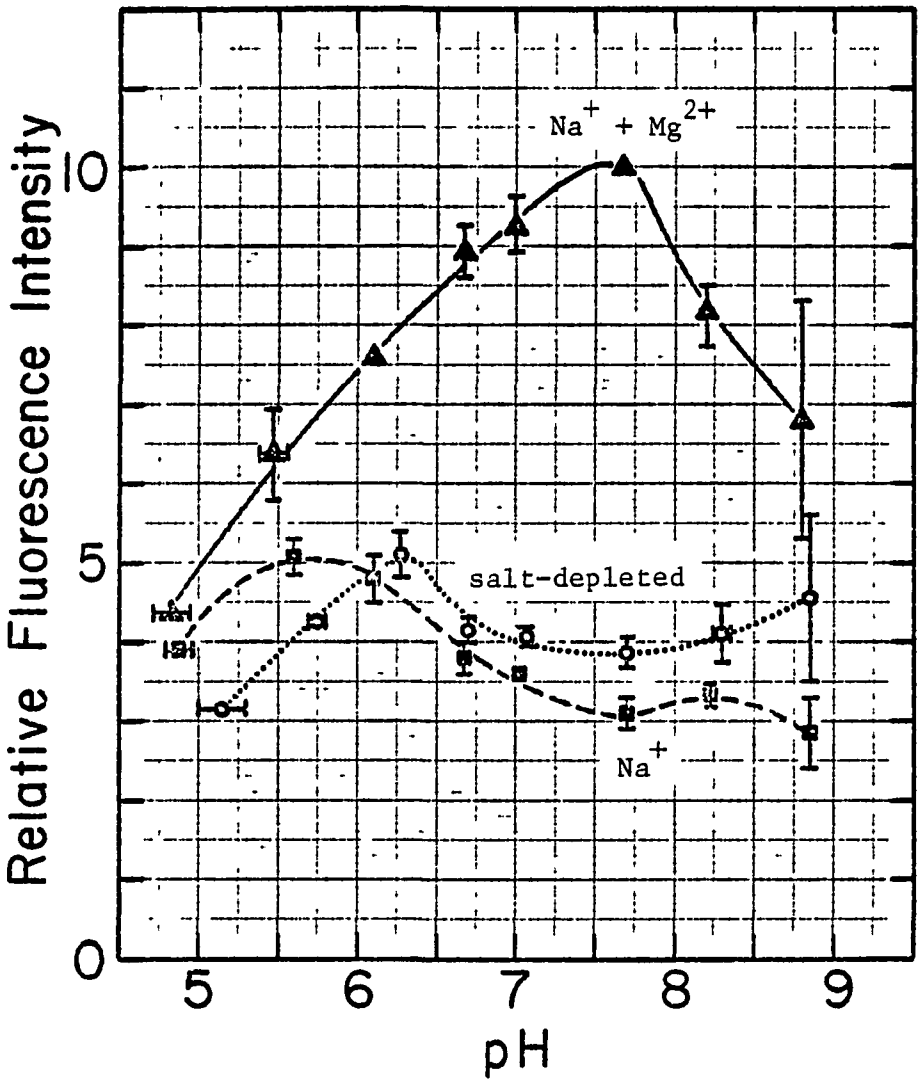

Figure 6.5 Relative Fluorescence Intensity at 685 nm versus pH for the Salt-Depleted, Na^+, and $Na^+ + Mg^{2+}$ Samples. Experimental conditions were similar to those in Fig. 6.1.

the addition of 10 mM Na^+, F_M shows a broad maximum centered around pH 5.8, intersecting the previous curve at pH ~6.1, so that $F_M(Na^+)$ is greater than F_M(cation-free) at lower pH's and smaller at higher pH's (closed squares). In the presence of Mg^{2+}, F_M peaks at pH 7.7, with F_M(pH 7.7) = $2F_M$(pH 5) ≃ $1.5F_M$(pH 8.8) (closed triangles).

The relative effects of Na^+ and Mg^{2+} addition on F_M over the pH range considered are shown as $F_M(Na^+)/F_M$(salt-depleted) and $F_M(Na^+ + Mg^{2+})/F_M(Na^+)$ in Fig. 6.6. Addition of 10 mM Na^+ to these salt-depleted thylakoids gave an ~20% increase in fluorescence at pH ~5.0 but an ~35% decrease at pH 8.8, the transition over the pH range being almost linear. The enhancement in relative fluorescence yield upon addition of Mg^{2+} to a sample containing Na^+ rises from ~10% at pH 5.0 to >300% at pH 7.8 followed by a slight drop to ~250% at pH 8.8.

FLUORESCENCE TRANSIENTS. To obtain an estimate of the relative contributions to the above changes from the "constant" and "variable" parts of fluorescence, the fluorescence transients at three pH's were measured. The results are summarized in Table 6.1. It is noted that F_o (the initial or "constant" fluorescence) is ~40% smaller at pH 7.8 than at pH 6.0, and ~25% larger at pH 8.9 than at pH 7.8. In the $Na^+ + Mg^{2+}$ sample F_o at the two lower pH's are about the same, but is ~20% smaller at the highest pH. A comparison at constant pH of the salt-depleted, Na^+, and $Na^+ + Mg^{2+}$ samples shows that F_o decreases by ~15% upon Na^+ addition to salt-depleted thylakoids, but increases by ~55% upon addition of Mg^{2+} only at pH 7.8 (cf. Wydrzynski et al., 1975), no change being observed at pH 6.1 and 8.9.

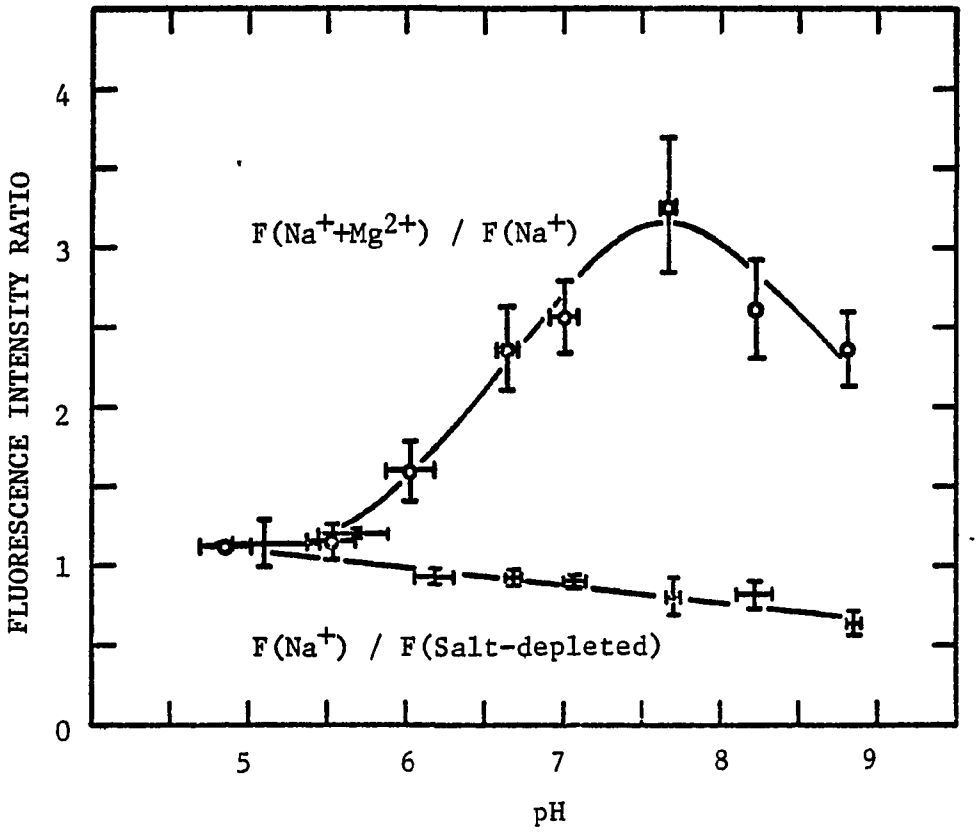

Figure 6.6 Fluorescence Intensity Ratios as Function of pH, Using the Data from Fig. 6.5.

TABLE 6.1

pH Dependence of the Cation Effects on the Initial and Maximum Relative Fluorescence at 23° C

	pH 6.1 ± 0.2		pH 7.8 ± 0.1		pH 8.9 ± 0.1	
Sample	F_o	F_M	F_o	F_M	F_o	F_M
Salt-depleted	10.0	20.7	5.7	12.7	7.1	17.0
10 mM NaCl	8.5	16.9	5.4	10.2	6.9	15.2
10 mM NaCl 10 mM $MgCl_2$	8.6	20.1	8.5	27.8	6.9	15.2

Fluorescence was detected at 685 nm through a monochromator (band-pass, 6.6 nm). Excitation was with broad-band blue light (white light through Corning CS 3-73 + CS 4-96 filters). [Chl] = 5 µg/ml. Other experimental details as given in Chapter 2.

The ratio of variable to maximum fluorescence as a function of pH, based on the data in Table 6.1, is given in Table 6.2. The same effects of cations on F_v/F_M as in the microsecond fluorescence transient induced by a single flash (Table 4.4) are observed at pH 7.8; i.e., a decrease in the ratio with addition of Na^+ and an increase with subsequent addition of Mg^{2+}. However, these effects are either diminished or absent at higher and lower pH's.

FLUORESCENCE LIFETIME. A direct measure of whether the relative fluorescence yield changes are changes in the efficiency of fluorescence is its lifetime. The pH dependences of the chlorophyll a fluorescence lifetimes for thylakoids suspended in cation-free, Na^+, or $Na^+ + Mg^{2+}$ medium are plotted in Fig. 6.7. For both the salt-depleted and Na^+ samples, the lifetimes show a general decline with increasing pH; some slight undulations in the pH profiles exist and may be indicative of structures poorly resolved in the present study. In the case of the $Na^+ + Mg^{2+}$ sample, the pH profile of the lifetime shows a broad peak around pH 7.5. The relative fluorescence intensities, which were simultaneously measured with the lifetimes, show the same pH profiles as the latter. This point is alternatively illustrated by the lifetime versus relative yield plots in Fig. 6.8 (same symbols as in Fig. 6.7). It is seen that in the steady-state the relative fluorescence intensity varies almost linearly with the lifetime as the latter changes with pH. Noteworthy is the fact that the pH profiles of lifetime of fluorescence in Fig. 6.7 closely resemble those from steady-state fluorescence intensity measurements in DCMU-treated thylakoids (Fig. 6.6). It seems,

TABLE 6.2

pH Dependence of the Cation Effects on the Ratio of Variable to Maximum Fluorescence

	$\frac{F_v}{F_M} = \frac{F_M - F_o}{F_M}$		
Sample	pH 6.1	pH 7.8	pH 8.9
Salt-depleted	0.52	0.55	0.58
10 mM NaCl	0.50	0.47	0.55
10 mM NaCl 10 mM $MgCl_2$	0.57	0.69	0.55

F_v/F_M was calculated from the data in Table 6.1.

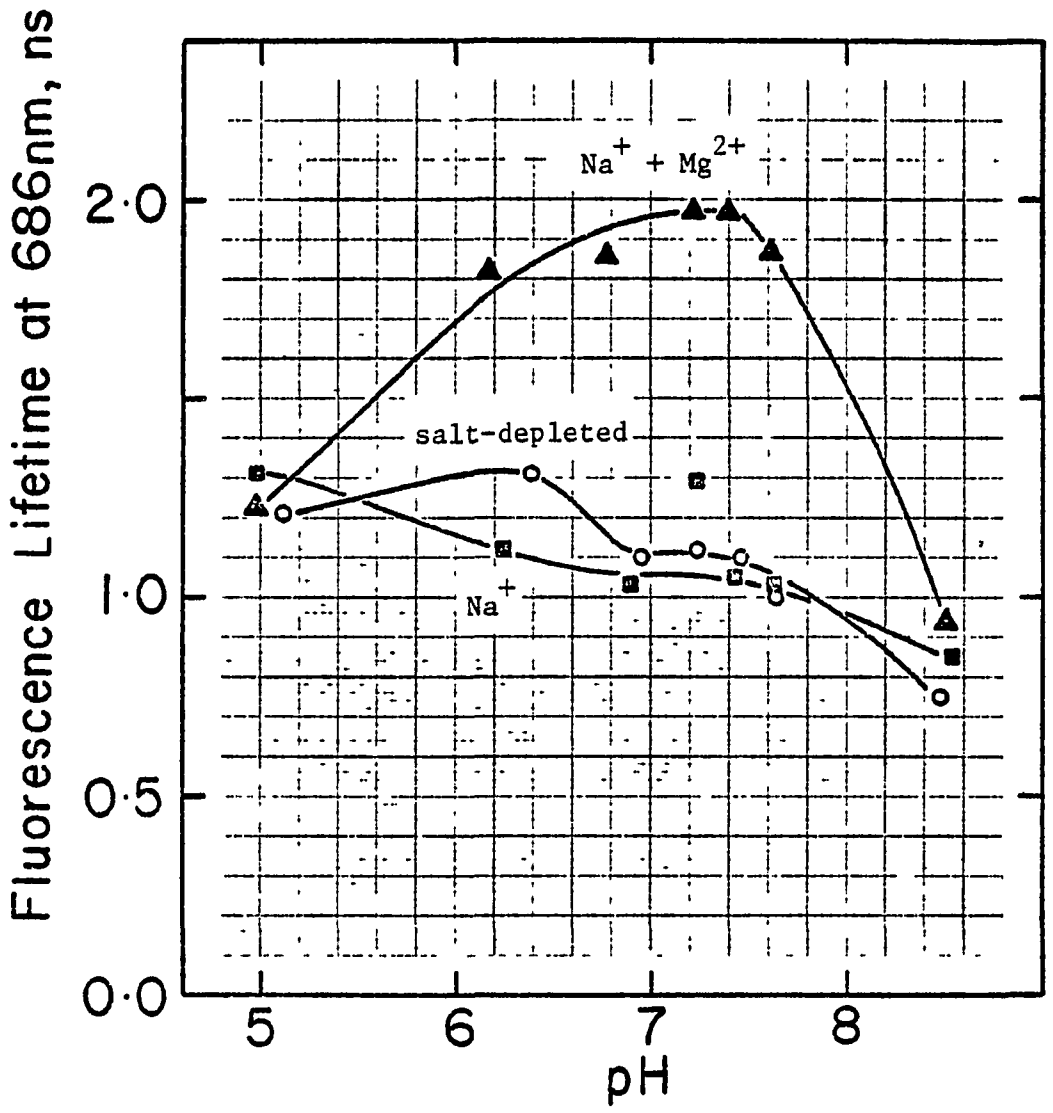

Figure 6.7 Fluorescence Lifetime as Function of pH for the Salt-Depleted, Na^+, and $Na^+ + Mg^{2+}$ Samples. Excitation was at 632.8 nm and fluorescence was detected through an interference filter at 686 nm (FWHM, 6.8 nm). [Chl] = 10 μg/ml; temperature = 20° C.

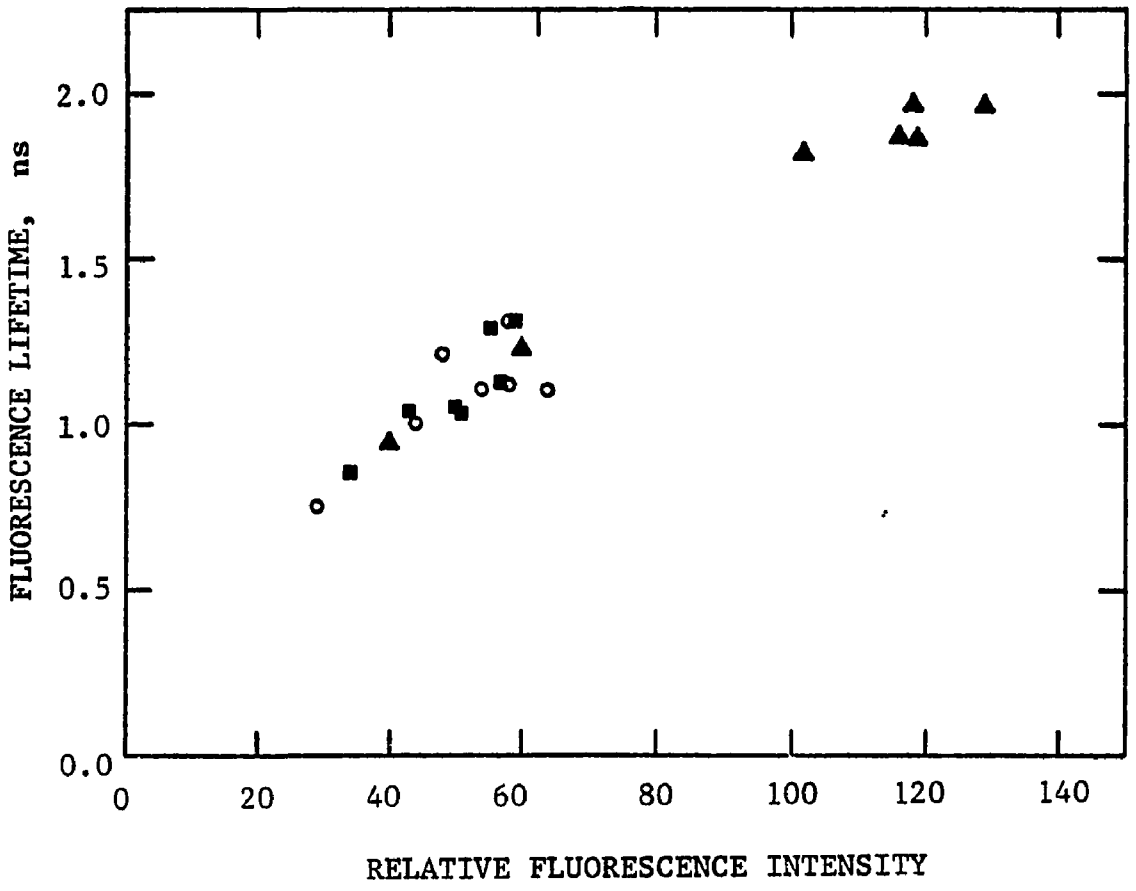

Figure 6.8 Fluorescence Lifetime versus Intensity as the Latter Changes with pH in the Three Cationic Conditions in Fig. 6.7. Open circles: salt-depleted; Solid squares: Na^+; Solid triangles: Na^+ + Mg^{2+}.

therefore, that the pH profiles of the steady-state fluorescence are unaffected by the presence of DCMU at the time the cations are added. It is important to note that, when DCMU is added to a sample fluorescing at the maximum level, a slow transient in the fluorescence may result, depending apparently on the pH and cationic conditions of the suspension medium. The addition of DCMU to $Na^+ + Mg^{2+}$ samples at about neutral pH first enhances the fluorescence slightly and then slowly decreases it, taking several minutes to complete and attaining a final fluorescence level ~20-30% lower than the initial "steady-state" level. This effect of DCMU was not pursued further as the conditions for its observation were not used throughout this study--DCMU when used was always added prior to cation addition, and in all instances the measurements were made at least 10 minutes after the thylakoids were incubated in the final suspension medium.

FLUORESCENCE POLARIZATION. The degree of polarization of fluorescence at 686 nm was measured at acid and alkaline pH's (Table 6.3). The results show that the Na^+-induced increase in the degree of polarization of fluorescence is greater at pH 6.6 (~11%) than at pH 9.0 (~4%). The Mg^{2+}-induced decrease of the polarization is about the same (10-13%) at both pH's. Increasing pH also leads to a general decrease in the degree of polarization of fluorescence.

6.3.1.3 pH Dependence of Cation Effects on Chlorophyll a Fluorescence at 77 K

FLUORESCENCE LIFETIMES. The P-level fluorescence lifetimes at 77 K were measured at 686, 695, and 730 nm for 3 different pH's--6.2,

TABLE 6.3

pH Dependence of Cation Effects on Chlorophyll *a* Fluorescence Polarization

Sample	pH	Degree of Polarization (%)
Salt-depleted	6.6	2.8
Na^+		3.1
$Na^+ + Mg^{2+}$		2.8
Salt-depleted	9.0	2.5
Na^+		2.6
$Na^+ + Mg^{2+}$		2.3

Samples were 3 ml thylakoid suspensions in 100 mM sucrose + 2 mM Tris-HNO_3, [Chl] = 5 µg/ml, and [DCMU] = 3.3 µM. NaCl and $MgCl_2$ were added to the final concentration of 5 mM. Excitation was at 630 ± 2.5 nm and fluorescence detected through a cut-off filter (Schott RG 665) and an interference filter at 686 nm (FWHM = 6.8 nm). The temperature was 24° C.

7.7, and 8.8 (Table 6.4). For the pH range investigated, the addition of Na^+ to salt-depleted thylakoids causes decreases in τ(F686) and τ(F596); these decreases are large at pH's 7.7 and 8.8 (30-40% for τ(F686) and 17-20% for τ(F695)) and small at pH 6.2 (1-9%). Subsequent addition of Mg^{2+} causes 61-87% increases in τ(F686) and 60-90% increases in τ(F695). The changes in τ(F730) are small (~15%) as previously found for pH 7.0. In addition to these specific changes there is a general decrease in the fluorescence lifetime at all wavelengths with increasing pH.

EMISSION SPECTRA. The cation effects on the emission spectra at 77 K were also measured at 3 different pH's. Since variations in sample geometry from ice crystal formation during freezing precluded the direct comparison of fluorescence intensities in these measurements, the spectra were normalized to their relative fluorescence lifetimes at 686 nm. Given in Fig. 6.9 are the first emission spectra plotted by the above procedure. The typical three-band structure of the spectra with maxima at 684 (PS II), 693-696 (PS II), and 735 nm (PS I) are obtained at all pH's and cationic conditions investigated. At pH 6.3, low concentrations of monovalent cations slightly enhance quanta redistribution from photosystem II to photosystem I, as a slight decrease in fluorescence at 684 and a slight increase at 735 nm are observed; divalent cations clearly inhibit the redistribution of energy from PS II to PS I, as a large increase in F684 and F693 and a large decrease in F735 are observed. At pH's 8.0 and 9.1, however, it is found that the addition of Na^+ causes a large decrease in the

TABLE 6.4

pH Dependence of Cation Effects on Chlorophyll a Fluorescence at 77 K

Sample	pH	Lifetimes, ns τ(F686)	τ(F695)	τ(F730)
Salt-depleted	6.2	0.56	0.71	2.34
Na^+		0.51	0.70	2.31
$Na^+ + Mg^{2+}$		0.83	1.12	2.51
Salt-depleted	7.7	0.36	0.56	2.08
Na^+		0.25	0.45	2.22
$Na^+ + Mg^{2+}$		0.54	0.85	1.93
Salt-depleted	8.8	0.35	0.58	1.75
Na^+		0.23	0.48	1.70
$Na^+ + Mg^{2+}$		0.43	0.81	1.89

Thylakoid suspensions in 100 mM sucrose + 2 mM Tris-HNO_3 were frozen in a 1 mm path-length demountable cuvette; [Chl] = 14 μg/ml; [NaCl] = [$MgCl_2$] = 9.8 mM. Excitation was at 632.8 nm (40 mW/cm^2) and front surface fluorescence was detected through a Schott RG 5 filter and a 686 nm IF (FWHM = 6.8 nm).

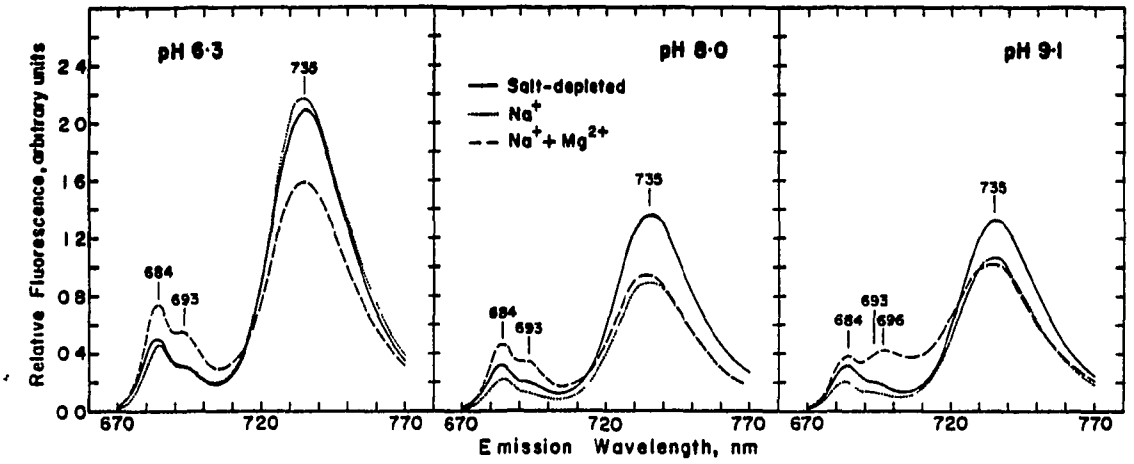

Figure 6.9 77 K Fluorescence Emission Spectra of Salt-Depleted, Na^+, and $Na^+ + Mg^{2+}$, at Three Different pH's. All spectra were normalized to their respective lifetime values (Table 6.4) at 684 nm. Excitation was at 636 nm (FWHM, 8 nm) and fluorescence was detected through a Schott RG 665 glass filter and a monochromator (band-pass 2 nm). All spectra were corrected for transmission characteristics of filter and monochromator, photomultiplier sensitivity, and stray light; details as given in Chapter 2. [Chl] = 10 μg/ml; $[Na^+] = [Mg^{2+}] = \sim 10$ mM.

relative fluorescence yield from all the three bands. Subsequent addition of Mg^{2+} causes a large increase in the fluorescence at 684 and 693-696 nm, but not that at 735 nm.

FLUORESCENCE TRANSIENTS. The fluorescence transients were studied with the purpose of evaluating the various energy distribution and redistribution parameters. Since this has been done for thylakoids at neutral pH (Tables 3.3 and 3.4), the study was made on samples at lower and higher pH's. The initial (F_o) and maximum (F_M) fluorescence at 690 and 730 nm at pH 6.2 and 8.8 are presented in Table 6.5; the fluorescence at 690 nm for the different samples are normalized according to their measured lifetimes by taking $\tau(F690) = [\tau(F686) + \tau(F695)]/2$. At the resolution used in these transient measurements, the fluorescence bands at 684 and 693 nm (see Fig. 6.9) appear as a single band with a peak at ~685 nm. As discussed in Chapter 3, the fluorescence at 690 nm monitors mainly PS II emission and that at 730 nm monitors mainly PS I emission. When a thylakoid sample is dark adapted and frozen, actinic illumination results in an initial fluorescence, F_o, followed by an exponential rise to a final maximum level F_M (cf. Fig. 2 in Butler and Kitajima, 1975a). Under normal operating conditions, PS I fluorescence is independent of the redox state of the PS I reaction center (Vredenberg and Slooten, 1967), and this "variable" fluorescence (~10-20% of total intensity at 730 nm) has been suggested by Butler and Kitajima (1975a) to result from energy transfer from PS II to PS I.

TABLE 6.5

Cation Effects on the Initial and Maximum Fluorescence at 77 K at 690 and 730 nm at Different pH's

Sample	pH	690 nm			730 nm	
		F_o	F_M	$\frac{F_v}{F_M}$	F_o	F_M
Salt-depleted	6.2	22.3	65.1	0.66	237.2	300.8
Na^+		29.5	62.1	0.53	277.0	339.2
$Na^+ + Mg^{2+}$		36.6	100.0	0.63	216.8	257.8
Salt-depleted	8.8	19.9	47.7	0.58	232.6	265.9
Na^+		24.0	36.4	0.34	204.9	225.0
$Na^+ + Mg^{2+}$		47.7	63.6	0.25	174.4	186.3

Thylakoid suspensions (0.5 ml) in 100 mM sucrose + 2 mM Tris-HNO_3 were adsorbed on two layers of cheese-cloth (average sample thickness, 0.3 mm) and frozen at 77 K. Excitation was through a 635 nm interference filter (FWHM = 8 nm), and fluorescence detected through a Corning CS 2-59 glass filter and a monochromator set at 690 nm (band-pass = 10 nm). Chlorophyll concentration was 20 µg/ml, [NaCl] = [$MgCl_2$] = 9.8 mM. Other technical details were as given in Chapter 2.

F_v/F_M at 690 nm is also presented in Table 6.5. This ratio, which, according to the analysis in Chapter 3, is the product of the efficiency of excitation transfer from the antenna to the reaction center of photosystem II and the efficiency of back-transfer from the closed center, may be taken as an index of the extent of excitation cycling between the antenna and the reaction center. At both acid and basic pH's the addition of 10 mM Na^+ to salt-depleted thylakoids lowers F_v/F_M at 690 nm. However, the subsequent addition of 10 mM Mg^{2+} while increasing F_v/F_M at acid pH, causes a further decrease in the ratio at basic pH. These results at pH 6.2 are similar to those found for room temperature transients; at pH 8.9, Mg^{2+} does not cause any change in F_v/F_M (Table 6.2).

EXCITATION DISTRIBUTION AND REDISTRIBUTION IN PHOTOSYSTEM II. From the above results of lifetimes and transients, estimates of the energy distribution and redistribution parameters in photosystem II were made using the dual-spillover model developed in Chapter 3. For these calculations the extreme values for the efficiency of excitation transfer from antenna to reaction center in photosystem II, $\eta_{T(22')}$, deduced from delayed light emission studies (Chapter 4) at pH 7.6, were used (last column in Table 6.6). The results are summarized in Table 6.6. The findings of major interest to this investigation are (1) that 10 mM Na^+ induces an ~8% decrease in β (the fraction of total absorbed quanta initially distributed to PS II) at acid pH and a slight increase (~4%) at alkaline pH, while 10 mM Mg^{2+} induces an ~13% increase in β at both acid and alkaline pH's, (2) that the Mg^{2+}-induced decrease

TABLE 6.6

Energy Distribution and Redistribution Parameters in Photosystem II Based on 77 K Fluorescence Transients and Lifetimes at Acid and Basic pH's

Sample	pH	β	η_{D2}	η_{F2}	$\eta_{T(21)}$	$\eta_{T(22')}$
Salt-depleted	**6.2 ± 0.1**	**0.53**	**0.17**	**0.01**	**0.12**	**0.70**
Na^+		0.49	0.16	0.02	0.16	0.66
$Na^+ + Mg^{2+}$		0.55	0.13	0.02	0.05	0.80
Salt-depleted	8.8 ± 0.1	0.45	0.21	0.01	0.08	0.70
Na^+		0.47	0.26	0.02	0.06	0.66
$Na^+ + Mg^{2+}$		0.53	0.15	0.03	0.02	0.80

The fraction of total absorbed quanta initially partitioned to PS II (β) and the efficiencies for de-excitation of excited chlorophyll in the antenna complex of PS II, namely, thermal dissipation (η_{D2}), fluorescence (η_{F2}), and energy transfer to PS I ($\eta_{T(21)}$) and reaction center II ($\eta_{T(22')}$), were calculated as described in Chapter 3 using the dual-spillover model and the results in Tables 6.4 and 6.5.

in the efficiency of energy redistribution from PS II to PS I ($\eta_{T(21)}$) is ~70% at both pH's. In addition, there is a significant Mg^{2+}-induced increase in the efficiency of fluorescence (η_{F2}) from PS II at alkaline pH. The sum of efficiencies of non-radiative processes other than energy transfer from PS II to PS I, $\eta_{D2} + \eta_{T(22')}$, is relatively constant among the samples at a given pH.

6.3.2 Mg^{2+} Effects on Electron Transport

The alternative means for investigating the effects of Mg^{2+} on the net electronic excitation distribution between the two photosystems is to study the partial electron transport rates through the two reaction centers under light-limiting conditions. Other effects of cations on the steps of electron transport from the site of primary charge separation to that of interaction between the electron transport chain and the artificial electron donor/acceptor may be studied by measuring the maximum or saturation rates of electron transport. An understanding of these effects may provide valuable insights into the role of the membrane surface charge density in influencing the cation effects.

6.3.2.1 Electron Transport in Light-Limiting Conditions

PS II PARTIAL REACTION: $H_2O \rightarrow$ DCPIP. Low intensities, high concentration of DCPIP used, and the slow steady-state measurements assured us that this was a purely PS II reaction under our experimental conditions (see Fork and Amesz, 1969). Light curves (rate of electron flow versus light intensity) at pH 7.3 were measured. For both samples with

and without Mg^{2+} added, the linear dependence of electron transport rate on light intensity in Fig. 6.10 ascertains that, at all levels of incident irradiance in these measurements, light was indeed limiting. This Mg^{2+} enhancement of the rate of the $H_2O \rightarrow$ DCPIP Hill reaction confirms most previous reports of the effects of this cation. Additional results in Table 6.7 extend the general validity of this effect to pH's 5.4 and 8.2.

PS I PARTIAL REACTION: $DCPIPH_2 \rightarrow MV$. The main aim here is to test if Mg^{2+} causes a reduction in the electron transport rate in photosystem I above pH 7.5 (cf. Bose, 1974; Rurainski and Mader, 1978) if $NADP^+$ is replaced by methyl viologen as electron acceptor. The results in Fig. 6.11 show that at both pH 7.0 and 8.2, Mg^{2+} causes only a decrease in the PS I electron transport rate.

6.3.2.2 Electron Transport in Saturating-Light Conditions

PS II PARTIAL REACTIONS: $H_2O \rightarrow Fe(CN_6)^{3-}$. High concentrations ($7.5 \times 10^{-4}$ M) of ferricyanide used assured us that this was a pure system II reaction (see Fork and Amesz, 1969). The effects of Mg^{2+} on the maximum rates of electron transport in the water to ferricyanide Hill-reaction are shown in Fig. 6.12. At pH < 7.8, Mg^{2+} enhances the electron transport rate, but, at pH > 7.8, Mg^{2+} inhibits this rate.

$H_2O \rightarrow DAD_{ox}$. Since ferricyanide has been shown to accept electrons from PS I under certain conditions, and may cause ambiguities in interpretation, measurements were made on a better defined PS II partial reaction, namely, water to oxidized diaminodurene (DAD_{ox}) in

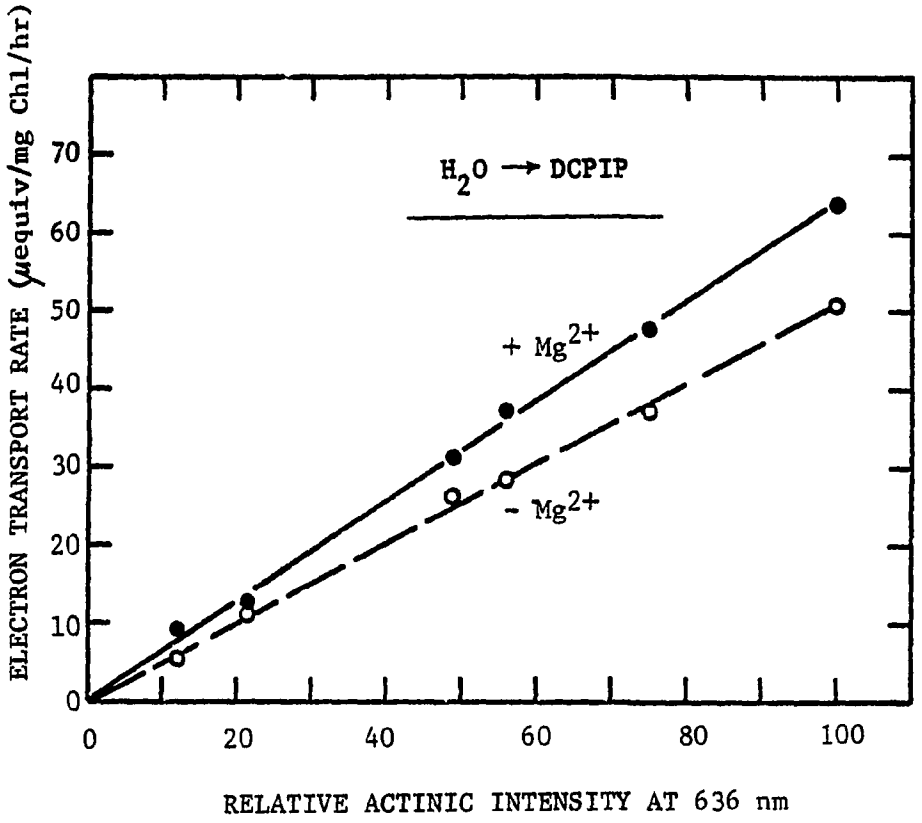

Figure 6.10 Rate of Electron Transport from H_2O to DCPIP as Function of Actinic Intensity. [Chl] = 10 μg/ml; [DCPIP] = 30 μM; [NH_4Cl] = 9.8 mM; [Mg^{2+}] = 9.8 mM; full actinic intensity = 22 mW/cm^2, pH = 7.3.

TABLE 6.7

Mg^{2+} Effect on $H_2O \rightarrow$ DCPIP Electron Transport Rates under Light-Limiting Conditions at Various pH's

pH	Actinic Intensity	Electron Transport Rates (μequiv./mg Chl/hr)	
		- Mg^{2+}	+ Mg^{2+}
5.4	75%	5.1	7.2
7.3	12%	5.4	9.0
	75%	37.1	47.7
8.2	12%	2.3	4.0

Rate of Electron Transport from H_2O to DCPIP as Function of Actinic Intensity. [Chl] = 10 μg/ml; [DCPIP] = 30 μM; [NH_4Cl] = 9.8 mM; [Mg^{2+}] = 9.8 mM; full actinic intensity = 22 mW/cm^2, pH = 7.3.

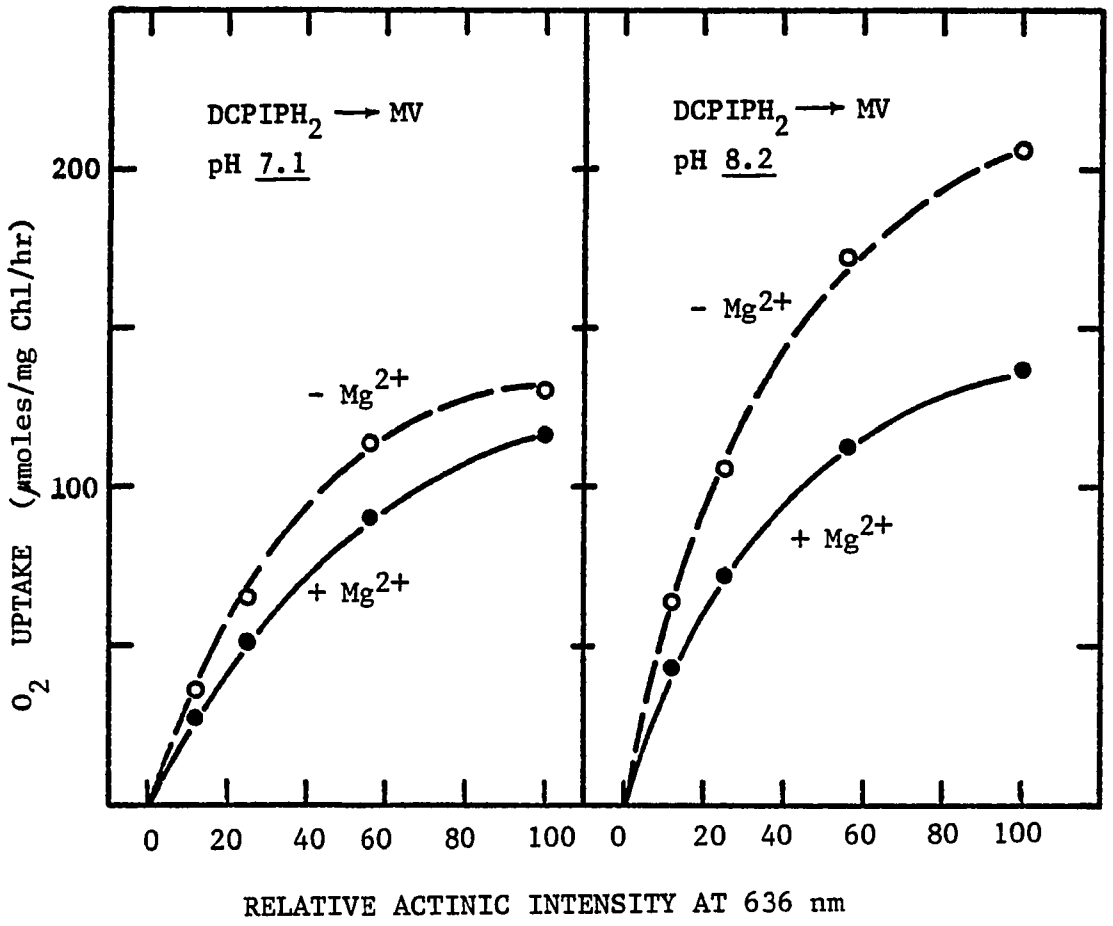

Figure 6.11 PS I Partial Electron Transport Rates in Limiting Light. [Chl] = ~25 μg/ml; [NH_4Cl] = 9.5 mM; [DCMU] = 4.8 μM; [DCPIP] = 60 μM; [Na ascorbate] = 1.9 mM; [MV] = 95 μM; and [$MgCl_2$] (when added) = 9.5 mM. Other details as given in Chapter 2.

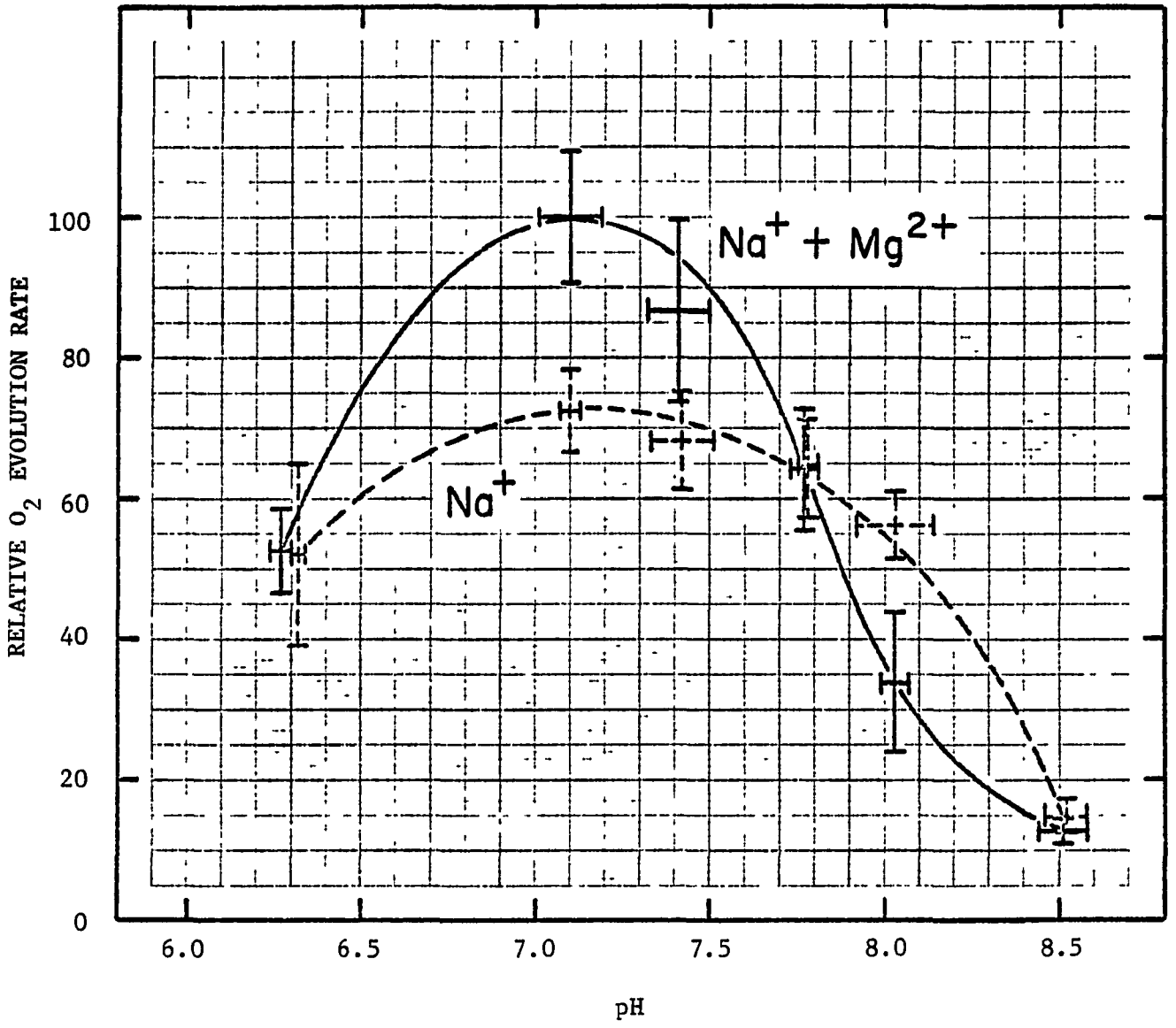

Figure 6.12 Normalized Electron Transport Rates from Water to Ferricyanide in Saturating Light. A relative rate of 100 denotes 144 μ moles O_2/mg Chl/hr. The results are the direct average of measurements from three different thylakoid preparations; the error bars denote one standard deviation. [Chl] = 25 μg/ml; [NH_4Cl] = 5 μg/ml; [$K_3Fe(CN_6)$] = 0.75 mM; when added, [$MgCl_2$] = 9.8 mM.

the presence of the plastoquinone antagonist dibromothymoquinone (DBMIB). As show in Fig. 6.13 very similar results to those of the $H_2O \rightarrow Fe(CN_6)^{3-}$ reaction were obtained confirming that the pH dependence of the Mg^{2+} effect is indeed not due to interference by the PS I partial reaction.

$H_2O \rightarrow$ DCPIP. The above results were not observed in this Hill reaction in the presence of DBMIB (Table 6.8), suggesting, perhaps, that in measurements under saturating light conditions, the pH profiles are those of the extent of interaction between the artificial electron acceptors and the electron transport chain. This study was not pursued any further.

PS I PARTIAL REACTIONS. Similar complexities as those of the PS II partial reactions at high light intensities also exist in some PS I reactions.

$DAD_{red} \rightarrow$ MV. The saturation rate for electron transport from DAD/ascorbate to methyl viologen are unaffected by Mg^{2+} at neutral and acid pH's. At pH 8.0 Mg^{2+} induces an ~39% enhancement (Table 6.8).

$DCPIPH_2 \rightarrow$ MV. pH sensitivity of the cation effect is observed in this reaction. Mg^{2+} induces a 5% increase in the electron transport rate at pH 7.1, but gives a 10% decrease at pH 8.2 (Table 6.8).

We emphasize again that the effects of cations on PS I and PS II reactions at saturating light need not be related to the excitation energy distribution and redistribution phenomenon and are most probably due to effects on various dark reactions. The effects of cations at low light intensities, presented above, are clearly related to the excitation energy distribution and redistribution; these results

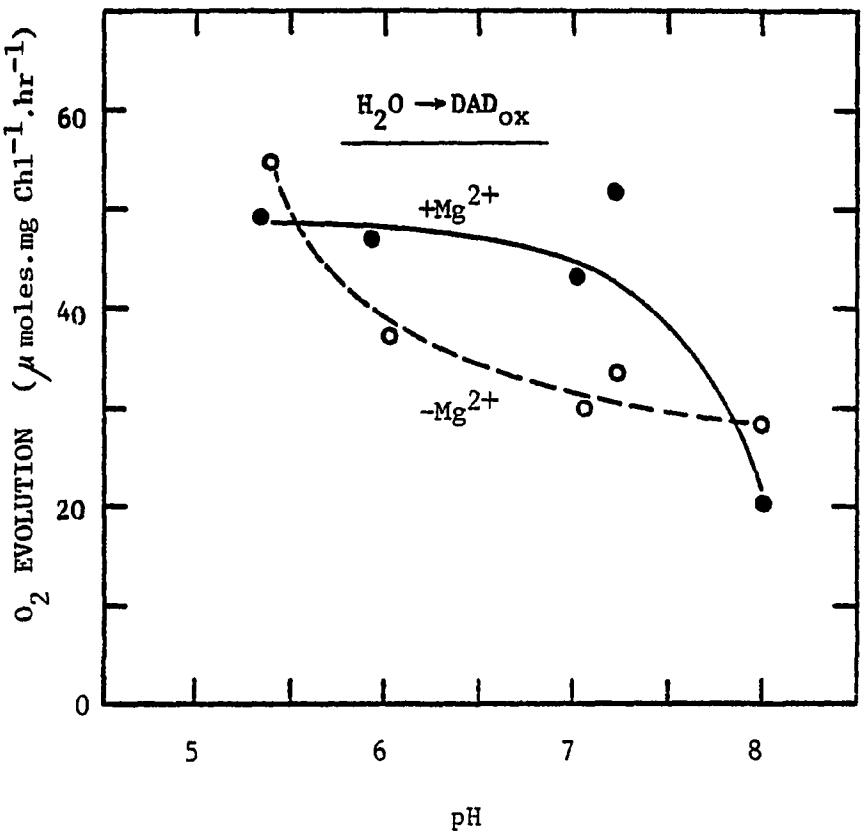

Figure 6.13 PS II Partial Electron Transport Rate in Saturating Light. [Chl] = ~25 µg/ml; [DBMIB] = 0.5 µM; [DAD] = 0.5 mM; [$K_3Fe(CN_6)$] = 9.7 mM; [NH_4Cl] = 5 mM; and $MgCl_2$ (when added) = 9.7 mM. Other experimental details were as given in Chapter 2.

TABLE 6.8

Mg^{2+} Effects on the Saturation Rates of Electron Transport in PS I and PS II Partial Reactions at Various pH's

Reaction	pH	Electron Transport Rates (μequiv./mg Chl/hr)	
		- Mg^{2+}	+ Mg^{2+}
PS II			
$H_2O \rightarrow$ DCPIP	6.4	248	216
(+ DBMIB)	8.3	257	243
PS I			
$DAD_{red} \rightarrow$ MV	6.3	205	208
	7.3	202	206
	8.0	223	309
$DCPIPH_2 \rightarrow$ MV	7.1	316	333
	8.2	630	569

PS II partial reaction; [Chl] = ~15 μg/ml; [NH_4Cl] = 9.6 mM; [DBMIB] = 0.5 μM; [DCPIP] = 60 μM; and [$MgCl_2$] (when added) = 9.6 mM. PS I partial reaction; [Chl] = ~25 μg/ml; [NH_4Cl] = 9.5 mM; [DCPIP] = 60 μM; [Na ascorbate] = 1.9 mM; [DAD] = 0.5 mM; [MV] = 95 μM; [DCMU] = 4.8 μM; and [$MgCl_2$] = 9.5 mM. Other details as given in Chapter 2.

confirm and extend the concept of regulation of excitation distribution by cations.

6.4 Discussion

6.4.1 General Comments

The large number of variable parameters and the multitude of functional dependences on pH for samples in different cationic media provide much complexity to the problem of evaluating this information. Biological variability, however, has been minimized by limiting all measurements to thylakoids prepared from three separate chloroplast isolation. Undoubtedly, a clear understanding of all the results is difficult at this time without further study. The main task here is to extract from these results those findings relevant to the questions posed in Chapter 1. To this end the discussion will be directed only to the explanation of those results of immediate interest.

The results in Fig. 6.1 show that the maximum cation effects are observed at ~10 mM cations over the pH range of interest. Thus, unless otherwise stated, it will be understood throughout this discussion that cations when added are to final concentrations of ~10 mM. The first point of interest is the pH range over which the conclusions in the earlier chapters regarding the role of Na^+ and Mg^{2+} in the initial distribution and redistribution of electronic excitation in and between the two photosystems are valid.

6.4.2 Steady-State Fluorescence

The results from DCMU-poisoned thylakoids in Figs. 6.5 and 6.6 show clearly that the Na^+-induced decrease in steady-state room temperature fluorescence is pH dependent--Na^+ induces a decrease in fluorescence only at pH > 6.1 but causes the opposite effect at pH < 6.1. Thus, earlier conclusions (Gross and Hess, 1973; Wydrzynski et al., 1975) are valid in pea chloroplasts only for pH > 6.1, and are best observed at alkaline pH. However, the Mg^{2+}-induced enhancement of fluorescence holds from pH 5 to pH 9 (Figs. 6.5 and 6.6), with the maximum effect around pH 7.5. Similar pH dependences of the room temperature fluorescence lifetimes are observed in thylakoids without DCMU treatment (Fig. 6.7). An almost linear dependence between the relative fluorescence intensity and its lifetime in simultaneous measurements of the two in these thylakoids is demonstrated in Fig. 6.8. This shows that the major effect is on the quantum efficiency of fluorescence. Comparison of the pH profiles of the lifetime for the salt-depleted and Na^+ samples raises some uncertainty as to the exact pH's where these curves cross. This problem does not exist between the $Na^+ + Mg^{2+}$ and the Na^+ samples. Thus, it is concluded with good certainty that the Na^+-induced inhibition of steady-state fluorescence exists between pH's ~6 and ~8, and that the Mg^{2+}-induced enhancement of fluorescence is valid over the pH range 5-9. Both effects are DCMU insensitive over their respective pH ranges.

6.4.3 Initial Distribution of Excitation Quanta

The initial distribution of excitation quanta into the two photosystems is obtained from the analysis of the parallel 77 K fluorescence lifetime and transient data (for details, see Chapter 3). The results (Table 6.6) show that Na^+ decreases β, the fraction of total quanta initially distributed to PS II, by ~8% at pH 6.2 and increases it by ~4% at pH 8.8. Mg^{2+}, on the other hand, increases β by 12-13% at both pH's. Along with the previous results at pH 7.0 (Table 3.4) it is concluded that β is decreased by Na^+ at pH's 6.2 and 7.0 and increased by Mg^{2+} over the pH range 6-9. This conclusion is consistent with the values of the initial fluorescence (F_o) at room temperature (Table 6.1) at pH 7.8, which confirm those of Wydrzynski *et al.* (1975), if F_o changes at 685 nm are attributed only to β changes.

6.4.4 Excitation Redistribution from PS II to PS I

The results in Table 6.6 also show that the Na^+-induced increase in the efficiency of excitation transfer from PS II and PS I occurs when the suspension medium is acidic (pH 6.2) but not when it is alkaline (pH 8.8). In conjunction with previous results (Table 3.4) at neutral pH, it is inferred that this effect of Na^+ occurs only at near-neutral pH's. The relative decrease in the efficiency of excitation transfer from PS II to PS I upon addition of Mg^{2+} to the Na^+ sample is ~70% at all pH's tested. The absolute efficiency, however, declines with increasing pH. Thus, the Mg^{2+} inhibition of excitation redistribution from PS II to PS I is pH insensitive in the range 6-9.

The above conclusions from 77 K fluorescence are supported at room temperature by results from two types of measurements. First, measurements of the degree of polarization of fluorescence at 686 nm (mainly PS II) in Tables 5.8 and 6.3 show an increase in polarization (less energy transfer in PS II) by Na^+ addition and a decrease by subsequent Mg^{2+} addition (greater energy exchange in PS II), and a decline in the Na^+ effect at high pH. These results are substantiated by those of the fluorescence polarization at 712 nm (Table 5.8)--F712 has a more significant contribution from PS I emission which shows opposite Na^+- and Mg^{2+}-induced changes on $\underline{P}$(F712) as for $\underline{P}$(F686). Second, the efficiency of fluorescence from PS II (which is in competition with non-radiative energy transfer from PS II to PS I) is directly measured by the lifetime of fluorescence at 686 nm. These lifetime results (Fig. 6.7) show a Na^+-induced decrease in efficiency of fluorescence for pH between ~6 and 8, and a Mg^{2+}-induced enhancement of this efficiency over pH between ~5.5 and ~8.5. These results are also in fair agreement with those of the cation effects on fluorescence intensity (Figs. 6.5 and 6.6).

Cation effects on the radiationless processes (including excitation redistribution from PS II to PS I) are inferred from the ratio of variable to maximum fluorescence (F_v/F_M) in the 77 K and room temperature transients (for details, see Chapters 3 and 4). This ratio has been discussed in two formulations earlier, and in each instant as the product of two terms which ultimately measures changes in the efficiencies of nonradiative processes in the antenna and closed reaction

center of photosystem II. What the relative roles of those radiationless processes are in the ratio F_v/F_M is unknown. The results, however, show that Na^+ causes a decrease in this ratio over the pH range 6.1-8.9 (Tables 6.2 and 6.4), while Mg^{2+} causes an enhancement at near-neutral pH's (6.1 and 7.8). Changes in the radiationless processes may cause the decrease in the maximum fluorescence efficiency at alkaline pH's (Fig. 6.7).

6.4.5 Net Distribution of Electronic Excitation

Changes in the net distribution of excitation quanta between the two photosystems are often demonstrated by changes in the rate of electron transport in PS I and PS II when light absorption is rate-limiting (cf. Barber, 1976; Williams, 1977). The results (Figs. 6.10 and 6.11 and Table 6.7) show that Mg^{2+} enhances the light-limited electron transport rates in PS II and inhibits those in PS I from pH 5.4 to pH 8.2. The results at about neutral pH confirm previous results (see Barber, 1976); the rest are new. The inadequacy of electron transport data is that they do not provide any information regarding the fractional contributions of initial quanta distribution and subsequent redistribution in the overall change.

The second objective of this investigation was to gain some insight into understanding the implications of the pH dependence of the Mg^{2+}-effect on electron transport from $DCPIPH_2$ to $NADP^+$, first made by Bose (1974) and recently reported by Rurainski and Mader (1978). Their experimental findings are that Mg^{2+} enhances the light-limited electron transport at pH < 7.5 and inhibits this rate at

pH > 7.5. This information has been used by Rurainski and Mader (1978) to question the hypothesis of Murata (1969) that Mg^{2+} inhibits excitation redistribution from PS II to PS I. The logic of Rurainski and Mader (1978) appears to be in error by the supposition that the Mg^{2+} inhibition of electron flow from $DCPIPH_2$ to $NADP^+$ is the key evidence for excitation "spillover." Though this may have been so at the time of proposal of the hypothesis, it is no longer true. Independent evidence now exists for the Mg^{2+} effect on electron transport from $DCPIPH_2$ to methyl viologen, from low intensity steady-state measurements on P700 oxidation (Henkin, 1976), and from steady-state measurements of electron paramagnetic resonance signal I in the presence of light absorbed primarily by one or both photosystems (Tikhonov et al., 1977). Since the only available information on the pH dependence of the Mg^{2+} effect on electron transport in PS I is for the $DCPIPH_2 \rightarrow NADP^+$ system, a logical approach to evaluating the argument advanced by Rurainski and Mader was to test the $DCPIPH_2 \rightarrow MV$ system for a similar pH dependence. It is found here that Mg^{2+} induces an ~35% decrease in the light-limited electron transport rates from $DCPIPH_2$ to methyl viologen in DCMU-treated thylakoids at pH 7.1 and 8.2 (Fig. 6.11). From these findings, it is suggested that the reported pH sensitivity of the Mg^{2+} effect on electron transport in the $DCPIPH_2 \rightarrow NADP^+$ system is peculiar to the use of $NADP^+$ as the terminal electron acceptor. It seems possible that the binding of ferredoxin to $NADP^+$ is not only Mg^{2+} sensitive (Harnischfeger and Shavit, 1974) but also pH sensitive. Complications involving the ferredoxin-$NADP^+$ acceptor-system have also previously been suggested

by Marsho and Kok (1974) when they noted that the Mg^{2+} enhancement of the quantum yield of the $DCPIPH_2 \rightarrow NADP^+$ reaction could be reversed by the prior presence of 30 mM monovalent cations.

The last point of concern for this investigation was the pH sensitivity of the cation effects. Since it is known that the thylakoid surface is negatively charged (see e.g., Nobel and Mel, 1966; Berg et al., 1974); Nakatani et al., 1978), H^+ should be expected to compete with cations if the effects of the latter are purely electrostatic (cf. Barber et al., 1977). This notion suggested the need for additional investigation into the influence of varying the pH on the cation effects, especially since it had previously been reported by Mohanty et al. (1972) that thylakoids from oat chloroplasts at 77 K showed an enhanced emission at 685 nm and a diminished emission at 735 nm when the pH was lowered to 3.8. The experimental information accumulated here is certainly insufficient to provide any conclusions but could serve as partial indicator of the areas needing investigation. Two points noted in this study seem to suggest a role of pH in regulating the cation effects.

(1) The apparent shift in the half-saturation concentration for divalent cations to lower concentrations with increasing pH (Fig. 6.2) may be indicative of a competition between H^+ and the divalent cations. The increasing effectiveness of monovalent cations with increasing pH (Fig. 6.1) may indicate the competition between H^+ and the monovalent cations.

(2) The pH dependence of the electrophoretic mobility of thylakoids (Nakatani et al., 1978) show an almost constant response between pH 6 and pH 10. However, the divalent cation effect on steady-state fluorescence (Figs. 6.5 to 6.7) shows a strong pH dependence from pH 6 to 9, suggesting a possible role for pH apart from simple surface electrostatics.

The effects of pH on primary processes are indeed complicated (see also Wraight et al., 1972). Aside from the knowledge that fairly acid (pH 4.5) and basic (pH 9.3) conditions inactivate the oxygen evolving apparatus (Pulles, 1978; Briantais et al., 1977; Wraight et al., 1972), and that the primary charge separation in P700 is insensitive to pH between 3 and 11 (Rumberg, 1964) very little else is known of the effects of pH on the natural events of photosynthesis in thylakoids. This is not surprising since without the advantage of a simpler purified system very little can be done to understand the pH profile of any individual process. Perhaps the possibility for comparison between samples could provide a useful alternative to obtaining some understanding of the effects of pH on thylakoid events.

6.5 Concluding Remarks

The present chapter has provided us with the following new information and conclusions:

(1) The optimum conditions for the observation of the Mg^{2+} effect on fluorescence are to detect the emission at 685 nm, with the sample containing 10 mM Mg^{2+} at pH ~7.5 (Figs. 6.1, 6.2, 6.4 to 6.7);

(2) The half-saturation concentration of the Mg^{2+} effect shifts to lower values with increasing pH (Fig. 6.2);

(3) The Mg^{2+} effect on fluorescence is emission wavelength dependent, with the maximum between 680 and 690 nm, a minimum between 710 and 720 nm;

(4) The room temperature fluorescence lifetime and its intensity are, to a first approximation, linearly related (Fig. 6.8);

(5) Mg^{2+} decreases the efficiency of excitation energy redistribution by ~70% from pH 6.2 to 8.8 (Table 6.6);

(6) Mg^{2+} enhances the light-limited electron transport rate through PS II in the pH range 5.4-8.2 (Fig. 6.10 and Table 6.7); the partial electron transport rate in PS I, in limiting light, is inhibited by Mg^{2+} at both pH's 7.1 and 8.2 (Fig. 6.11);

(7) The antagonistic effects between low concentrations of Na^{+} and Mg^{2+} hold only in the pH range 6-8, in pea thylakoids.

CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

7.1 The Project

Homann (1969) first reported that cations enhance the room temperature chlorophyll a fluorescence intensity in the presence or absence of the electron flow inhibitor DCMU. Murata (1969) showed that Mg^{2+} increases and decreases the light-limited electron transport rate in PS II and PS I, respectively, and also enhances the ratio of PS II/PS I emission at 77 K. Since then, the effects of cations on steady-state chlorophyll a fluorescence (room and low temperature) and light-limited electron transport rates in thylakoid membranes have been extensively studied. At the time this investigation was undertaken, a number of conflicting views prevailed on the interpretation of these cation effects. The hypothesis that cations decrease excitation "spillover" from PS II to PS I (Murata, 1969) was questioned by Hoch and Rurainski (1972) who favored the concept of reaction center II activation by Mg^{2+}. Sun and Sauer (1972) suggested that cations enhanced "spillover" of energy from PS I to PS II. Marsho and Kok (1974) favored the occurrence of cation regulation of initial quanta distribution (see also Bonaventura and Myers, 1969; Duysens, 1972) for explanation of their electron transport results, while Jennings and Forti (1974) and Malkin and Siderer (1974) suggested that cations alter non-radiative processes competing with fluorescence. The possibility for the multiple effects of cations had been mentioned by Marsho and

Kok (1974), Malkin and Siderer (1974), and Wydrzynski et al. (1975), and treated more quantitatively by Butler and Kitajima (1975a). The situation was confusing because of the lack of general agreement on the processes regulated by cations.

The strategy taken for this project was to study the cation effects on the primary photoprocesses of photosynthesis by directing investigation at the following questions:

(1) Which primary photoprocesses are regulated by cations?

(2) What is their relative importance in the measured cation effects?

(3) Do measurements at room temperature and at 77 K reflect the same processes?

(4) What molecular events are involved in some of these changes?

The sucrose-washing method of Gross (1971) for cation depletion from thylakoids was chosen because of the possibility for studying antagonistic effects of mono- and di-valent cations in the same preparation (see Chapter 1 for references).

7.2 Which Primary Photoprocesses are Regulated by Cations?

According to the plan presented above, the initial task of the present study was to determine which processes are affected by cations. Briefly stated, our findings show that the initial distribution of absorbed quanta between the two photosystems, the redistribution of quanta absorbed in PS II to PS I, and some non-radiative transitions in PS II are simultaneously affected by cation addition to sucrose-washed (i.e., salt-depleted) thylakoids. The initial charge

separation, however, is independent of the presence of low concentrations of cations in the suspension medium, but the rate of recovery of the PS II reaction center is enhanced by Mg^{2+} (Table 7.1).

7.2.1 Initial Distribution of Quanta

Evidence in support of a Na^{+}-induced increase in the fraction of total absorbed quanta initially distributed to PS II comes from the following:

(a) Parallel evaluation of the 77 K fluorescence lifetime and transient data (Tables 3.3, 3.4, and 6.6) shows that β decreases by ~6% with Na^{+} addition at pH's 6.2 and 7.0 and increases by ~15% with subsequent Mg^{2+} addition over the pH range 6-9. The Mg^{2+} effect at about neutral pH confirms the previous findings of Butler and Kitajima (1975a). The results for the Na^{+} effect and the cation effects at different pH's are reported for the first time.

(b) The sensitization of PS II primary photochemistry as monitored by the light saturation curve for 100 μs delayed light emission shows an ~6% decrease with Na^{+} addition and an ~20% increase with subsequent Mg^{2+} addition (Fig. 4.7). These results are new.

(c) The relative change in the degree of polarization of fluorescence at 730 or 760 nm (at room temperature) upon addition of Mg^{2+} to the Na^{+} sample, expressed as fraction of $\underline{P}(Na^{+})$, when plotted as a function of excitation wavelength (Fig. 5.4) shows minima at ~650 and ~675 nm. Simultaneously, the ratio of the fluorescence intensity of the $Na^{+} + Mg^{2+}$ sample to that of the Na^{+} sample as function of excitation wavelength (Fig. 5.5) shows relative maxima at ~650 and ~675 nm.

These results show that Mg^{2+} enhances the efficiency for energy transfer from Chl LH to Chl a_{II}, thus providing the first direct experimental evidence for the Mg^{2+} regulation of this molecular event in thylakoids.

7.2.2 Excitation Redistribution

The evidence in support of the Mg^{2+} inhibition of excitation energy redistribution from PS II to PS I comes from the following:

(a) At 77 K, the fluorescence intensity quotient F690/F730 shows a larger increase by Mg^{2+} than the quotient of corresponding lifetimes, τ(F686 or F695)/τ(F730) (Table 3.2). The fluorescence lifetime at 730 nm shows very minor variations with Mg^{2+} addition, suggesting that the Mg^{2+}-induced increase in τ(F686 or F695)/τ(F730) reflects mainly the increase in efficiency of PS II fluorescence, interpreted here as a consequence of a decrease in the efficiency of excitation transfer from PS II to PS I, $\eta_{T(21)}$. A decrease in $\eta_{T(21)}$ gives an increase in F686 or F695 and a corresponding decrease in F730 explaining the greater increase in the quotient of intensities than of lifetimes. This approach is new.

(b) The analysis of the low temperature fluorescence lifetime and transient data (Tables 3.3 and 3.4) shows that $\eta_{T(21)}$ decreases by ~70% upon addition of Mg^{2+} to a sample containing ~10 mM Na^{+}. Results at pH's 6.2 and 8.8 show the same effect of Mg^{2+} on $\eta_{T(21)}$ (Table 6.6) providing experimental confirmation of the general validity of this Mg^{2+} effect over the pH range 6-9.

(c) At room temperature, the Mg^{2+} enhancement of fluorescence intensity as a function of the emission wavelength shows a relative

maximum at ~685 nm (PS II) and a minimum between 710 and 720 nm (PS I) (Fig. 6.4). The change in F686 has also been shown to correspond to the change in the PS II fluorescence lifetime, and, hence, its efficiency, over the pH range 6-9 (Fig. 6.5, 6.7, and 6.8).

(d) Qualitatively, the addition of Mg^{2+} to a thylakoid suspension containing Na^{+}, the degree of polarization of fluorescence, at room temperature, at 686 or 730 nm decreases, but that at 712 nm increases (Table 5.8). This effect at 686 nm has been confirmed over the pH range 6.6 to 9.0 (Table 5.8 and 6.3). The combination of these new results in (c) and (d) provide the most definitive evidence, to date, for the Mg^{2+} inhibition of PS II to PS I excitation energy redistribution.

(e) The inverse effects of Mg^{2+} on the light-limited electron transport rates of PS I and PS II are confirmed at pH ~7.5 (cf. Table I in Barber, 1976), and extended over the pH range 5.5-8.2--addition of Mg^{2+} inhibits the PS I light-limited rates ($DCPIPH_2 \rightarrow MV$) by 11-35% (Fig. 6.11), but enhances the PS II rates ($H_2O \rightarrow DCPIP$) by 30-75% (Fig. 6.10 and Table 6.7). These effects on PS I electron transport are similar to those reported by Bose (1974) and Rurainski and Mader (1978) for pH < 7.5, but contradictory to their results for pH > 7.5 which we suggest reflect the complexities of the $NADP^{+}$ system, used by these authors, rather than those of the Mg^{2+} effect.

(f) Supportive evidence comes also from study of the room temperature fluorescence transient. A simple analysis of the fluorescence transient in terms of three parallel pathways competing for electronic

excitation captured by the antenna chlorophylls of PS II--namely, fluorescence, non-radiative de-excitation in the antenna (thermal dissipation or energy transfer to PS I), and non-radiative dissipation by an open or closed reaction center--gives the ratio of variable to maximum fluorescence [F_v/F_M, equivalent to $1 - (\frac{F_o}{F_M})$] as the product of (i) the relative decrease in the efficiency of excitation energy dissipation by a closed PS II reaction center relative to an open one; and (ii) the yield of PS II primary photochemistry (Chapter 4). Within the pH range 6.0-9.0 (see Tables 4.4 and 6.2) the room temperature fluorescence transients both in the μs and ms time-scales show that F_v/F_M decreases with the addition of Na^+; subsequent addition of Mg^{2+} increases this ratio at acid and slightly alkaline pH's, but the change is absent at pH 8.8. Analysis of these results with the aid of data from the 515 nm absorption change (Fig. 4.8) and fluorescence lifetime measurements (see Discussion in Chapter 4) provided the conclusion that in the absence of Mg^{2+} the radiationless deexcitation of excited chlorophyll in the antenna complex of PS II is greater than in its presence. These radiationless events include energy transfer from PS II to PS I, internal conversion, and intersystem crossing.

7.2.3 Reaction Center II Activation

Finally, the result against the possibility that Mg^{2+} increases the total number of PS II reaction centers capable of undergoing primary light-induced charge separation is the following: The initial amplitude of the flash-induced absorbance change at 515 nm--an indicator of the

amount of charge separation in PS II when P700 is kept oxidized by ferricyanide (see Chapter 4 for details)--is unaffected by the addition of Mg^{2+} (Fig. 4.8), confirming an independent report by Vredenberg and Schapendonk (1978) that came to our attention after the completion of our experiments. The rate of recovery of the 515 nm absorbance change is enhanced more by Mg^{2+} in thylakoid suspensions in the absence of DCMU than in its presence (see Fig. 4.8), suggesting a possible role of electron flow on the reducing side of PS II in the recovery of the 515 nm signal, and the effect of Mg^{2+} on it.

7.3 What is the Order of Importance of the Processes Affected by Cations?

From the quantitative information available, the largest relative change occurs in the Mg^{2+} inhibition of the efficiency of excitation transfer from PS II to PS I (~70%; Tables 3.3, 3.4, and 6.6). The next consistent effect is the Mg^{2+}-induced enhancement in the relative sensitization of PS II processes (~20%; see previous section). The Na^{+} effects on the above two processes show the same order of importance in the narrower pH range where they occur.

7.4 Do Room Temperature and 77 K Measurements Reflect the Same Processes?

The discussion for the first question reveals that the conclusions regarding the cation effects on initial excitation distribution and redistribution are derivable for measurements both at room temperature and 77 K. This is important because it shows that previous inferences,

based on low temperature measurements are valid at physiological temperatures. Our conclusions here regarding the Mg^{2+} inhibition of radiationless dissipation of excitation energy at the closed reaction center of PS II and the cation independence of the maximum photochemical capacity of reaction center II are both based on room temperature studies.

7.5 What Molecular Events are Involved?

The only molecular event that can be stated with definitiveness is the increased excitation transfer from Chl LH to Chl $\underline{a}_{II}$ by Mg^{2+}; this is based on the excitation spectra of fluorescence enhancement and degree of polarization change of chlorophyll a fluorescence at 730 and 762 nm (Figs. 5.4 and 5.5). Mg^{2+} causes an increase in depolarization of Chl a fluorescence when excitation is in Chl b (peak at 650 nm) or in Chl a 670. Taking the absorption of Chl a and Chl b in LHC to be at 670 and 650 nm, respectively, with Stokes' shifts of 10 nm and Chl $\underline{a}_{II}$ absorption to be at 680 nm, the Förster overlap integral is found to be quite favorable (see Shipman and Housman, 1978), suggesting that the regulation of energy transfer may involve changes in the distance and/or orientation factors (κ^2) between Chl LH and Chl $\underline{a}_{II}$. Both factors may be related to the Mg^{2+}-induced greater structural coupling between the light harvesting chlorophyll protein complex and the chlorophyll a antenna complex of PS II (Arntzen and Ditto, 1976). Interestingly, based on recent studies on the Mg^{2+}-induced spectral shift of the electrochromic effect in thylakoids, Vredenberg and Schapendonk (1978) have suggested that carotenoid orientations in the

thylakoid membrane are affected by cations. How carotenoid reorientations affect Chl LH is as yet unknown.

7.6 New Method for Estimation of Energy Distribution and Redistribution in Single Samples

By combining low temperature fluorescence transient and lifetime data we have developed a new method for estimating the initial distribution and redistribution of excitation quanta between the two photosystems in a single suspension of thylakoids (Chapter 3). This development represents an improvement over the original method of Butler and Kitajima (1975a) where two samples are needed (see also Analysis 2 in Chapter 3), and an alternative to that of Strasser and Butler (1977a) using 77 K transient and transmittance data. The main advantage of our method over the latter is the added possibility for obtaining the numerical values of the rate constants of different processes.

7.7 General Comments

In the present study we have investigated the effects of cations on chlorophyll a fluorescence and electron transport and their pH dependences in thylakoids. It is clear that the effects of Na^+ are not simply the reverse of those of Mg^{2+}. For instance, at room temperature, low Na^+ concentrations (~10 mM) at sufficiently low pH (≲5) produce the same effects as Mg^{2+} on steady-state fluorescence intensity, the pH range over which many of the Na^+ effects operate is narrower than that of the Mg^{2+} effects, and at high concentrations (~100 mM) Na^+ again produces the same effect on fluorescence as Mg^{2+}. The Na^+

effects have been neglected in many of our measurements and it is hoped that the available guidelines for investigation established in the present study would enable a more fruitful attempt at understanding the Na^+ effects in the future. Another aspect of interest for future experiments is the disappearance of the cation effects at low pH ($\lesssim$5). Although this may reflect the competition between H^+ and cations in modifying the surface electrostatics of the thylakoid membrane, it is suggested that it also reflects additional non-electrostatic effects of H^+ on thylakoids. Throughout this investigation the salt-depleted sample, when compared to the Na^+ sample, is found to qualitatively resemble many of the fluorescence characteristics of the $Na^+ + Mg^{2+}$ sample at sub-optimal concentrations of Mg^{2+}; the pH profiles of the steady-state fluorescence intensity and lifetime (Figs. 6.5 and 6.7) between the samples are, however, significantly different. This may be an indication that the extent of residual divalent cation binding in sucrose-washed thylakoids is pH dependent. Finally, it must be mentioned that although the present study has firmly established the multiplicity of effects of Mg^{2+} on photosynthetic processes in thylakoids, and although light-induced Mg^{2+} translocation across the thylakoid from the loculus to the stroma is known to exist (cf. review by Barber, 1976), there is no experimental evidence as yet to show that <u>in vivo</u> regulation of electronic excitation between the photosystems occurs by variation in Mg^{2+} concentration on one or the other side of the thylakoid membrane. Particularly interesting is the resemblance of <u>State I</u> and <u>State II</u> adaptation in algal cells and intact chloroplasts (cf. Barber, 1976;

Williams, 1977) to the case of thylakoids in the presence and absence, of Mg^{2+} respectively, because no evidence exists to show that Mg^{2+} translocation *in vivo* differentiates between light absorbed by PS I or PS II. Thus, at this time, the physiological significance of the cation effects to photosynthesis *in vivo* is still unknown.

TABLE 7.1

Conclusions Made in this Thesis

Conclusions	Results	Locations
1. Mg^{2+} induces an increase in the transfer of electronic excitation from PS II to PS I at 296 and 77 K	Mg^{2+} induces a greater increase in the PS II to PS I fluorescence intensity ratio than the lifetime ratio, and has very little effect on the lifetime of PS I fluorescence	Table 3.2
	Analysis of transient and lifetime of fluorescence from PS II and PS I shows that Mg^{2+} decreases, by ~70%, the efficiency of excitation transfer from PS II to PS I at 77 K (pH 6-9)	Tables 3.3, 3.4, 6.6
	The Mg^{2+}-induced increase in fluorescence yield has a relative maximum at ~685 nm (PS II) and a minimum at 710-720 nm (PS I) at 296 K (pH 6-9)	Figs. 6.3, 6.4
	*Na^{+} increases the degree of polarization of fluorescence at 685 nm (PS II) and decreases that at 712 nm (PS I); this effect is reversed by Mg^{2+}	Tables 5.8, 6.3
	Mg^{2+} inhibits the light-limited electron transport rates in PS I (pH 5.4-8.2), and enhances those in PS II at both pH 7.1 and 8.2	Figs. 6.10, 6.11 Table 6.7
	The ratio of variable to maximum fluorescence a 685 nm at 296 K is increased by Mg^{2+}	Table 6.2

Table 7.1 (Continued)

	Conclusions	Results	Locations
2.	Cations regulate the initial distribution of excitation quanta between PS I and PS II at both 296 and 77 K	Na^+ decreases (by 4-8%, pH 7 or less) and Mg^{2+} increases (by 11-17%, pH 6-9) the fraction of absorbed quanta initially distributed to PS II, based on the analysis of 77 K fluorescence lifetime and transient data.	Tables 3.3, 3.4, 6.6
		Analysis of the light saturation curve for delayed light emission at 100 μs shows an ~6% decrease by Na^+ and ~20% increase by Mg^{2+} of the sensitization of PS II primary photochemistry.	Fig. 4.7
3.	The regulation of initial distribution of excitation to PS II involves the better energetic coupling of Chl *a* and Chl *b* in LHC with Chl a_{II}.	** The ratio excitation spectrum for fluorescence at 730 and 762 nm, at 296 K, shows relative maxima at ~650 and ~675 nm, when the sample containing Mg^{2+} is compared with that without Mg^{2+}. Simultaneously the relative depolarization of fluorescence between these samples shows maxima at ~650 and ~675 nm.	Figs. 5.4, 5.5
4.	Mg^{2+} *does not* diminish the maximum capacity for primary charge separation in sucrose-washed thylakoids, but affects its recovery in normal electron flow.	An increase in the light-induced absorbance change at 515 nm by Mg^{2+} is not observed The decay of this 515 nm absorbance signal is enhanced by Mg^{2+} in samples untreated with DCMU.	Fig. 4.8

*Most definitive evidence to date for regulation of excitation redistribution.

**First direct evidence in thylakoids of energy transfer from the light-harvesting chlorophyll *a/b* protein complex to chlorophyll *a* of the PS II antenna complex in the regulation of excitation distribution by Mg^{2+}.

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1. Effects of Lead Ions on Photosystem I in Isolated Chloroplasts: Studies on the Reaction Center P700. Photosynthetica 10 (1976) 241-254.

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