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MAGNESIUM ION EFFECTS ON PRIMARY PROCESSES IN CHLOROPLAST PHOTOSYNTHESIS

Bruce Michael Henkin
(Ph.D. thesis)

June 1975

Prepared for the U.S. Energy Research and
Development Administration under Contract W-7405-ENG-48

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MAGNESIUM ION EFFECTS ON PRIMARY PROCESSES IN CHLOROPLAST PHOTOSYNTHESIS

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MAGNESIUM ION EFFECTS ON PRIMARY PROCESSES IN CHLOROPLAST PHOTOSYNTHESIS

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ABSTRACT

Through measurements of Mg^{2+} effects on chloroplast photochemistry and fluorescence we have attempted to define the nature of the Mg^{2+} interaction with photosynthetic energy transfer and electron transfer processes. In untreated chloroplasts, Mg^{2+} increases background (dead) fluorescence by about 30%. This increase saturates at a Mg^{2+} concentration of about 0.5 mM. By contrast, variable (live) fluorescence is stimulated about 250% and saturates at a Mg^{2+}-concentration of about 2.5 mM. These results indicate that the Mg^{2+} effect on live fluorescence has a different origin than the Mg^{2+} effect on dead fluorescence. To account for these different effects of Mg^{2+} on the two components of chloroplast fluorescence we have proposed two models for the arrangement of PS II pigment arrays associated with chloroplast fluorescence. According to one model, the two site model, live fluorescence is both sensitized and emitted by a different pigment array than dead fluorescence. In the other model, the single site model, separate live and dead pigment arrays sensitize the two components of emission which come from a common

fluorescent pigment array. At low actinic intensities, we observe that the variable fluorescence in the presence of DCIP is directly proportional to the actinic intensity for chloroplasts with or without Mg^{2+}. Under these conditions the variable fluorescence level of Mg^{2+}-containing samples is uniformly twice as large as the level for samples without Mg^{2+}. In DCMU and dithionite poisoned chloroplasts we find that Mg^{2+} approximately doubles the level of total fluorescence.

Analysis of our DCMU and DCIP experiments according to the models presented above leads to two conclusions:

1. Mg^{2+} increases in live fluorescence are most simply explained by a Mg^{2+}-induced increase in the effective size (absorption cross section) of the PS II live pigment array.

2. Mg^{2+} stimulation of live fluorescence cannot be accounted for by Mg^{2+} inhibition of energy transfer from PS II → PS I. The observation of a marked (∿70%) stimulation of the light-limited rate of PS II photochemistry (H_2O → DCIP) by Mg^{2+} supports our proposal that Mg^{2+} increases the effective size of the PS II live pigment array. (Our analysis also indicates that Mg^{2+} stimulation of light-limited PS II photochemistry cannot be explained by Mg^{2+} inhibition of energy transfer from PS II → PS I.)

In the case of PS I, the effect of Mg^{2+} on light-limited photochemistry is sensitive to the choice of terminal electron acceptor. For the PS I reaction, DCMU/Asc/TMPD → $NADP^+$, we find that Mg^{2+} significantly increases (∿50%) the light-limited rate of PS I photochemistry. In the case of the PS I reaction, DCMU/Asc/TMPD → methyl viologen/O_2, addition of Mg^{2+} causes a 25% decrease in the rate of oxygen uptake. On the basis of these results, it is not yet clear whether Mg^{2+} effects

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on light-limited PS I photochemistry are related to Mg^{2+} effects on PS II photochemistry and fluorescence, or whether other factors, such as PS I cyclic electron flow, are of significance in the interpretation of these experiments.

We have also examined the effect of Mg^{2+} on PS I electron transport at high light intensities. For the $H_2O \rightarrow$ methyl viologen, $H_2O \rightarrow NADP^+$, DCMU/Asc/Pcy $\rightarrow NADP^+$, and DCMU/Asc/Pcy $\rightarrow$ methyl viologen systems, we find that Mg^{2+} causes a significant (~30-60%) decrease in the photostationary level of oxidized P700. We also observe Mg^{2+} stimulation of $NADP^+$ reduction with either H_2O or Asc/Pcy as the electron donor. These results are interpreted in terms of Mg^{2+} stimulation of non-cyclic electron flow. Our results suggest that a site of Mg^{2+} control of photosynthetic electron transport exists between the site of Asc/Pcy donation to PS I and Y, the electron donor to Fd and methyl viologen.

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Acknowledgements

The research described in this thesis was performed under the supervision of Professor Kenneth Sauer. Although I am grateful to Professor Sauer for his numerous suggestions and criticisms which contributed to the success of this project, I am most deeply indebted to him for teaching me the necessity of a critical and analytical approach to scientific problems. I recognize now that his insistence on an unbiased critical approach to scientific research made an important contribution to my scientific development.

Although I wish to thank all the members of my research group that I have worked with for their comradeship and advice, I feel that there are certain individuals who deserve a special acknowledgement for their friendship and aid in scientific and non-scientific areas. Consequently, I wish to especially acknowledge the contributions and friendship of Alex Sun, Jerry Babcock, Jim Ellenson, Bob Blankenship, Doug Vaughan, and Sandy Asher. Outside of my research group, I wish to thank Steve Leone and Jack Finzi for the friendship and advice they provided me with during my years at Berkeley.

I am indebted to many of the staff and support personnel at the Laboratory of Chemical Biodynamics for the many ways that they

have helped me during my years as a graduate student. In particular, I wish to express my special thanks to Evie Litton, Jo Onffroy, Dick O'Brien, Mike Press, and Bill McAllister for the many things they did which made my tenure in the laboratory more pleasant.

Last, but certainly not least, I am deeply indebted to my parents for the constant encouragement that they have given me during my years of graduate study. This support and encouragement was a particularly valuable aid in combating and overcoming the frustrations which often arise during the course of scientific research.

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Abbreviations

Asc	Sodium ascorbate
ATP	Adenosine triphosphate
Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DCIP	2,6-Dichlorophenolindophenol, oxidized form
$DCIPH_2$	2,6-Dichlorophenolindophenol, reduced form
Fd	Ferredoxin
HEPES	(N'2-Hydroxyethyl-piperazine-N')-2-ethanesulfonic acid
MV	Methyl viologen
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
Pcy	Plastocyanin
PS II	Photosystem II
PS I	Photosystem I
RC II	Reaction center II
RC I	Reaction center I
TMPD	N,N,N',N'-Tetramethyl-p-phenylene diamine, oxidized form
$TMPDH_2$	N,N,N',N'-Tetramethyl-p-phenylene diamine, reduced form

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Dedication

To my Parents

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Chapter I

INTRODUCTION

Early History: Over two centuries have elapsed since the discovery by Priestly in 1771 that plants possess the ability to transform "phlogistic air" (carbon dioxide) into "dephlogistic air" (oxygen), yet much still remains to be explained about the mechanism of green plant photosynthesis. Ingenhousz (1779) identified the requirements of light and green material (chlorophyll) for photosynthesis. Senebier (1783) spelled out the importance of carbon dioxide in photosynthesis, while de Saussare (1804) emphasized the significance of water in photosynthesis. Mayer (1845) recognized the importance of photosynthesis as a means of utilizing solar radiation to form a reservoir of chemical potential which is consumed in later reactions.

Through the work of these and other scientists one could, by the 1930's, summarize the processes involved in green plant photosynthesis by the following equation:

$$CO_2 + H_2O \xrightarrow[\text{chlorophyll}]{h\nu} (CH_2O) + O_2$$

Hill (1939) observed oxygen evolution from isolated chloroplasts in the presence of ferric oxalate without fixation of carbon dioxide. He concluded that the problem of oxygen evolution could be studied separately from the problem of carbon reduction.

The work of Calvin and Bassham in the post World War II period elucidated the path of carbon in photosynthesis. Subsequently Trebst et al.[1] showed that the reduction of CO_2 to carbohydrate (sugar) is basically a "dark" process which requires two compounds

(NADPH and ATP) that are produced by light. It is therefore customary to refer to the reactions involved in the formation of assimilatory power (NADPH and ATP) as the primary (photochemical) processes of green plant photosynthesis. These primary processes are separated from the dark processes of carbon reduction spatially within the chloroplast. The enzymes responsible for CO_2 fixation are primarily localized in the aqueous region of the chloroplast interior known as the stroma (Fig. I.1), whereas the processes of $NADP^+$ reduction and photophosphorylation take place in the lamellar membrane structures called thylakoids.

The research discussed in this thesis was carried out on isolated broken (class II) chloroplasts which are capable of $NADP^+$ reduction but not carbon fixation. Hence, further discussion will be limited to the primary photochemical processes of photosynthesis and will omit any references to the reductive pentose phosphate cycle.

In order to understand the mechanism of $NADP^+$ reduction and ATP synthesis by chloroplasts, it is first necessary to discuss briefly the concepts of the <u>electron transfer chain</u> and the <u>photosynthetic unit</u>.

<u>Electron Transfer Chain</u>: Although we can represent the overall stoichiometry of $NADP^+$ reduction by the equation

$$H_2O + NADP^+ \xrightarrow[\text{chlorophyll}]{h\nu} 1/2\ O_2 + NADPH + H^+$$

this tells us little about the reaction mechanism and intermediates involved in the process. Hill and Bendall[2] suggested that electron transport from water to pyridine nucleotide could best be explained

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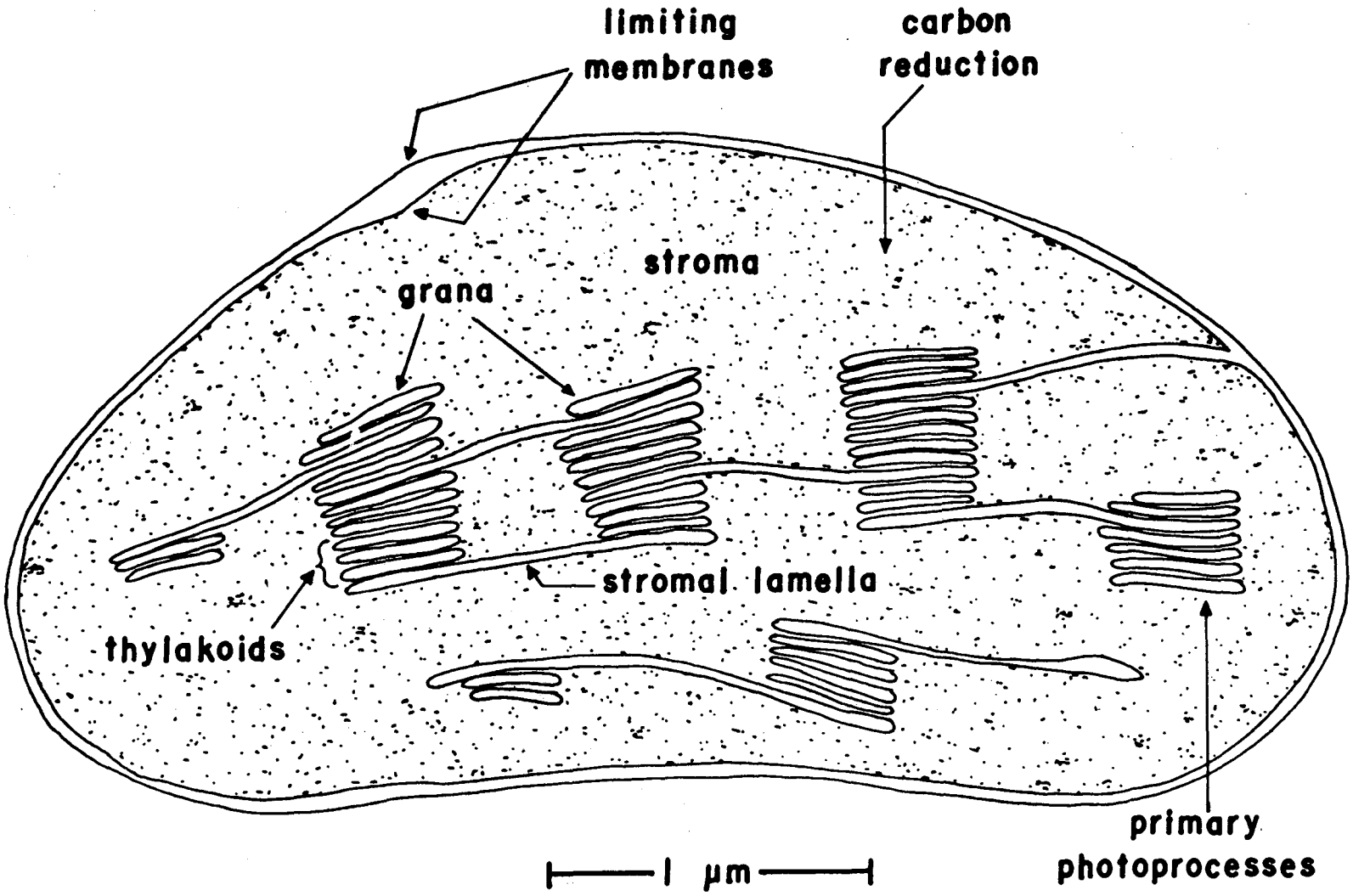

Figure I.1. Generalized chloroplast from a leaf mesophyll cell. XBL753-5122
(Adapted from P.S. Nobel, Plant Cell Physiology, Freeman, San Francisco, p. 18, 1970.)

by a model in which there are two light driven reactions operating in series and connected by a dark electron transport chain involving cytochromes, quinones, and other intermediates. Research conducted in the past decade has refined this model, now known as the Z scheme, to the level pictured in Fig. I.2. As seen in Fig. I.2, we have two chlorophyll photo-sensitized oxidation-reduction reactions and three segments of the "dark" electron transfer chain. Photosystem II is the site of oxygen evolution; the actual process by which water is "split" to yield molecular oxygen is not yet well understood. Kok et al.[3] have suggested that there are at least four as yet unidentified intermediates, known as the S states, which participate in the transfer of electrons from H_2O to the photoactive chlorophyll associated with Photosystem II (PS II). This specialized chlorophyll is a part of the PS II reaction center, RC II. Recent studies[4] indicate that electron transfer from water to RC II requires the presence of manganese, which is apparently bound to the chloroplast membrane. Inhibition and reactivation[5,6] experiments show that is is possible to "break" the electron transfer chain between H_2O and RC II and use artificial electron donors to replace H_2O as the ultimate electron donor to RC II. Electrons are transferred from RC II to an unknown compound called Q or C550 in a photochemically driven step. RC II is then reduced by electrons from water via the pathway described above. Electrons are transferred from the reduced form of Q (Q^-) via a thermodynamically favored ("downhill") series of dark electron transfer steps through a series of electron carriers including quinones, cytochromes and the blue copper protein plastocyanin to the photoactive chlorophyll of Photosystem I (PS I). This specialized trap chlorophyll is contained

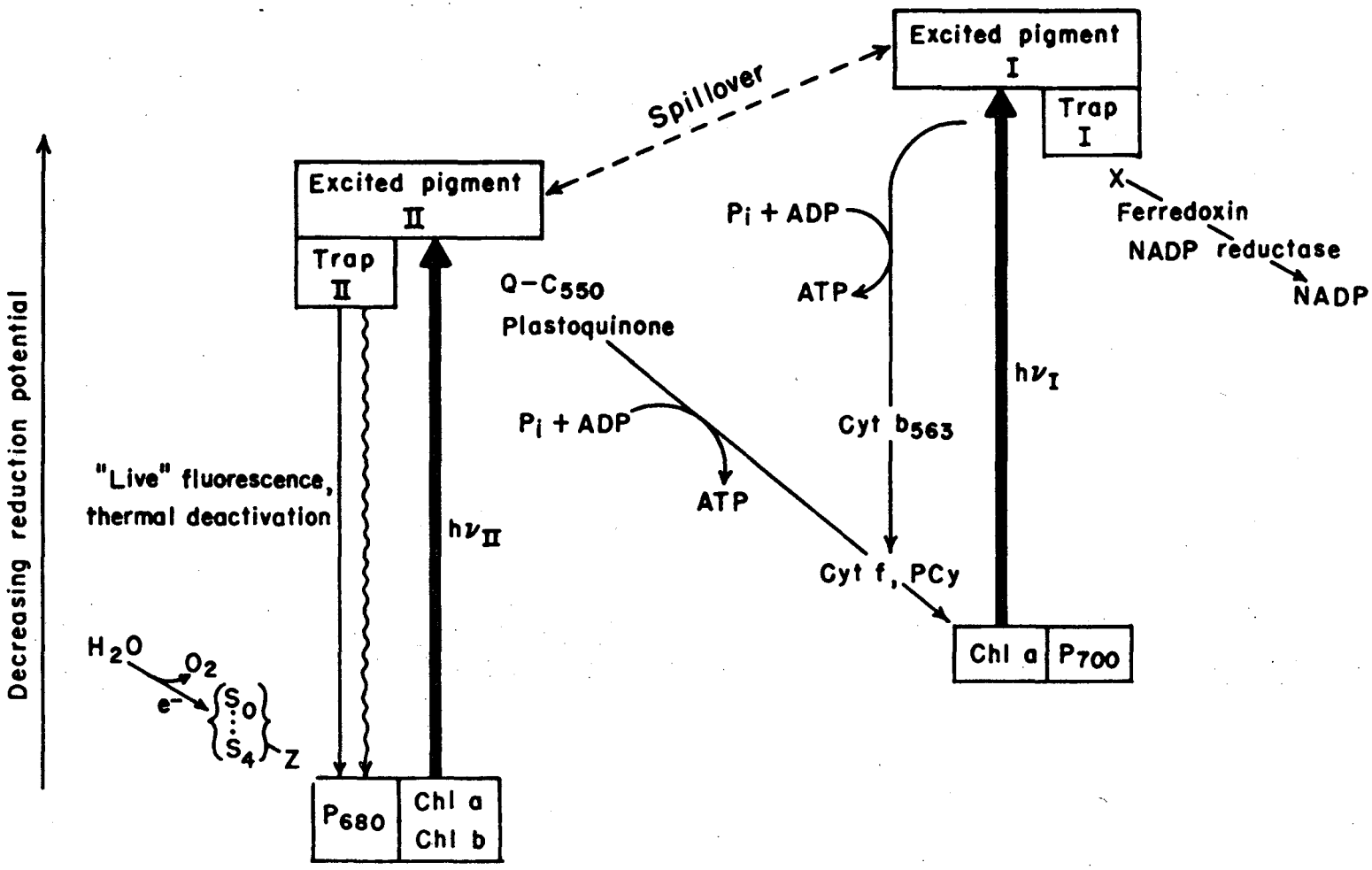

Figure I.2. Z scheme model for photosynthetic electron transport. See text for details. (Figure adapted from reference 16).

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in the PS I reaction center (RC I) and is known as P 700. Photoexcitation of P 700 results in the transfer of an electron to an unknown acceptor (X or P 430). Electrons are then transferred from X in another series of "downhill" steps through the non-heme iron sulfur protein ferredoxin, the flavoprotein enzyme ferredoxin-NADP reductase to the terminal acceptor, $NADP^+$.

Reaction Centers and Antenna Pigments: The photoactive chlorophylls associated with RC II and RC I do not directly absorb most of the energy which they utilize in the redox reactions that they photosensitize. Instead, these reaction centers act as collectors or traps of energy which is absorbed by a much larger array of light harvesting pigments (mainly chlorophyll a, but also including chlorophyll b, carotenoids and other pigments). The reaction centers are present in a concentration of about one reaction center/400 chlorophyll molecules. The light harvesting pigments plus the reaction center form what is termed a photosynthetic unit. More formally, we can define a photosynthetic unit (PSU) as smallest group of collaborating pigment molecules sufficient to effect a photochemical act. The earliest experimental evidence supporting the concept of a PSU came from classic experiments of Emerson and Arnold.[7] Using brief saturating flashes they observed that if the time between flashes was less than 40 milliseconds there was a decrease in the yield of O_2 produced per flash. Even more importantly, they observed 1 O_2 evolved/ 2500 chlorophylls excited. This ratio indicated that there is a relatively small number of molecules involved in the primary photochemical process surrounded by a large array of light gathering molecules

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capable of transferring their excitation to the reaction center. Subsequently Gaffron and Wohl[8] showed that under dim light if each chlorophyll was a reaction center it would take about one hour for a chlorophyll in the leaf to absorb sufficient quanta to produce the four oxidizing equivalents needed to form O_2 from H_2O. Since O_2 is evolved rapidly (within seconds) after the start of illumination, this implies cooperation of absorbed photons through a PSU. Support for a model of a PSU where there is about 1 reaction center/400 chlorophylls comes from inhibitor studies, concentration of intermediate electron carriers, and chloroplast fragmentation studies.

Energy Transfer and the PSU: The exact mechanism by which excitation energy is transferred from the antenna array to the reaction center has been a subject of considerable theoretical speculation.[9] Some models[10] have proposed that the efficiency of energy transfer between adjacent molecules is inversely proportional to third power of the distance (r) between two molecules. Other models[11] have proposed that the efficiency of energy transfer is proportional to r^{-6}. At the present time it is not possible to distinguish between these proposed mechanisms experimentally.

In addition to transfer of energy to the trap, antenna molecules can be de-excited by other non-radiative and radiative (fluorescence) pathways. Fluorescence emission, usually attributed to PS II at room temperature, provides information about energy transfer[12] and electron transfer associated with PS II.[5] Liquid nitrogen temperature measurements have been used to study energy transfer associated with PS I as well as PS II.[12] The theoretical basis of chloroplast fluorescence will be discussed in detail in Chapter II.

In addition to the possibility of energy transfer between antenna molecules and trap within a PSU, one must consider the possibility of energy transfer among adjacent PSUs. Recent studies[13,14] indicate that chloroplasts prepared under normal conditions exhibit energy transfer between PS II PSUs. As discussed below, we must also consider the possibility of energy transfer between PS II and PS I PSUs as well as the possibility of energy transfer between adjacent PS I PSUs.

Interactions between PS II and PS I: It is clear from the discussion of the Z scheme that PS II and PS I interact on the level of electron transport. However, it is also possible for the two photosystems to interact at the level of transfer of excitation energy. Two extreme models have been summarized by Myers.[15] One model, known as the separate package model, postulates that no energy transfer is possible between PSUs associated with PS II and those associated with PS I. At the other extreme, we have the spillover model in which excitation absorbed by a PSU in one photosystem is transferred to the other photosystem.

These models may be distinguishable on the basis of measurements, in the separate experiments at the same wavelength, of the quantum yield of a "pure" PS II reaction (i.e., one which involves PS II alone) or of a "pure" PS I reaction. If the sum of these two (zero intensity) quantum yields is greater than unity, it favors some form of the spillover model. A quantum yield of 1 or less cannot distinguish between the two models.[16] Although in early studies the quantum yield sum was less than unity, more recent investigations,[16,17] where sums of 1.5 to 2.0 were obtained, definitely support the spillover model. One version

of the spillover model is represented in Fig. I.2. This formulation, specifies distinct PS II pigment arrays which transfer excitation to RC II and distinct PS I antenna pigment arrays which transfer excitation to RC I. When the spillover mechanism is operative, excitation absorbed by PS I pigments can be transferred to PS II pigments and eventually to RC II. Similarly in the presence of spillover, RC I can utilize excitation which was initially absorbed by PS II pigments. There have been several attempts[12,17,18] in recent years to determine what factors control the distribution of photons between the two types of pigment systems. At present, the nature of the control mechanism is still unknown and is a subject of great controversy.[17-20]

Separation of Photoreactions: Following the original suggestion of Hill and Bendall that chloroplast electron transport involved two photoreactions connected by a series of dark reactions, there were many attempts to define the properties of these photochemically driven reactions experimentally. Losada *et al.*[21] showed that it was possible to separate the overall $H_2O \rightarrow NADP^+$ process into two partial reactions which could be studied separately. Using an indophenol dye (DCIP), Losada *et al.* separated the $H_2O \rightarrow NADP^+$ sequence into two reactions, $H_2O \rightarrow DCIP$ and $DCIPH_2 \rightarrow NADP^+$ and studied the properties of these partial reactions. In this study, the $H_2O \rightarrow DCIP$ reaction evolved oxygen but did not form ATP. It was therefore believed to involve only one photochemical step and was associated with what is now known as PS II. The $DCIPH_2 \rightarrow NADP^+$ reaction not only reduced $NADP^+$, it also produced ATP and proceeded even in the presence of the oxygen evolution inhibitor DCMU. For these reasons this reaction was assigned exclusively

to what is now known as PSI. The subsequent observation[16] of a quantum yield of unity for the $H_2O \rightarrow$ DCIP reaction strongly supported the earlier concept that this reaction involved only PS II. A unit quantum yield was also observed for the $H_2O \rightarrow$ ferricyanide reaction.[17] (The observation of a unit quantum yield indicates not only the existence of a single photochemical step in the reaction under study but also indicates that under the experimental conditions employed all quanta absorbed by both photosystems are being utilized by a single reaction involving only one of the photosystems.)

The Hill and Bendall assignment of the $H_2O \rightarrow NADP^+$ reaction as a reaction which requires participation of both photoreactions is consistent with the quantum yield of 0.5 reported by several[16,17] laboratories. Although the $DCIPH_2 \rightarrow NADP^+$ reaction has a quantum yield of only 0.5-0.6 in the red region of the spectrum[16] ($\lambda \leq 680$ nm), at longer wavelengths ($680 \leq \lambda \leq 730$ nm) quantum yields of unity can be obtained. The unit quantum yield obtained with far red illumination implies the involvement of a single photochemically driven step (PSI). Reactions which involve PS II differ in their wavelength dependence from those which involve solely PS I. Reactions of the "pure" PS I type proceed fairly efficiently in red light ($\phi \cong .5$) and show an increase in quantum yield in far red light ($\phi \approx 1$). By contrast, reactions which involve PS II, such as $H_2O \rightarrow$ DCIP or $H_2O \rightarrow NADP^+$ proceed efficiently only in red light and show a marked decrease in quantum yield beyond 680 nm. This decrease in quantum yield was first seen in algae by Emerson and Lewis in 1943 and is known as the "red drop". The wavelength dependence of the quantum yields suggest that only shorter wavelength quanta are sufficiently

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energetic to sensitize PS II reactions while PS I reactions can be sensitized by either short or long wavelength excitation. Strong evidence for the different wavelength characteristics of the two photoreactions was obtained by Duysens et al.[22] in their study of the photo-oxidation and photoreduction of cytochrome f in algae. They showed that long wavelength illumination (680 nm) caused photo-oxidation of cytochrome f, and subsequent illumination at 560 nm caused the cytochrome to become photo-reduced. In the presence of DCMU, Duysens et al. observed that short (λ = 560 nm) wavelength illumination caused the cytochrome f to be photo-oxidized. These "antagonistic effects" of short and long wavelength light led these authors to suggest that in chloroplasts there are two sequential photoreactions with different spectral sensitivities. The photo-induced redox changes observed for cytochrome f suggest that it is located on an electron transport pathway between the photoreactions. The quantum yield and action spectra measurements discussed above are extremely significant, because they provide us with methods to study PS II behavior independent of PS I and vice versa.

In addition to the experiments cited above, the phenomenon of enhancement, first seen by Emerson and co-workers in the 1950's, played an important role in advancing our knowledge of the properties of the photoreactions involved in chloroplast electron transport. Emerson noted that the rate of photosynthesis (of algae) when illuminated simultaneously with red and far-red light was greater than the sum of the rates obtained for red and far-red illumination separately. $E = \frac{Rate_{R + FR}}{Rate_R + Rate_{FR}}$. Enhancement can also be measured as the increase

in quantum yield of a reaction at one (red or far-red) when the sample is simultaneously illuminated by another (far-red or red) wavelength. Enhancement has also been observed in isolated chloroplasts for the $H_2O \rightarrow NADP^+$ reaction.[17,18] Duysens et al.[22] suggested that enhancement could be interpreted in terms of two sequential photoreactions with different spectral response characteristics. In this view, when far-red illumination alone is present the overall rate of $NADP^+$ reduction is limited by an insufficient amount of excitation reaching RCII. When supplementary red light is added to the far-red illumination this limitation is removed and consequently the overall efficiency of electron transport ($NADP^+$ reduction) increases. In the presence of simultaneous red and far-red illumination there may also be a redistribution of short wavelength quanta between the photosystems so as to allow optimum use of these quanta by the two photoreactions.

In addition to the photochemical and biochemical methods discussed above, subchloroplast particles which show behavior characteristics of PS I or PS II can be isolated.[23,24] Particles which have almost "pure" PS I activity have been prepared by several different techniques.[23,24] Other fractions are enriched in PS II activity although they usually retain a depleted amount of PS I activity.

Over the past decade the two light reaction model of chloroplast electron transport has been attacked by proponents of a single light reaction model (Vennesland[25] and a three light reaction model (Knaff and Arnon).[26] At the present time, however, the two light reaction model is the hypothesis most clearly in accord with the vast majority of experiential data from laboratories throughout the world.

Magnesium Ion Effects on Photosynthesis: At the conclusion of this discussion of current concepts of energy transfer and electron transport in photosynthesis, it is appropriate to review previous studies on the influence of Mg^{2+} on primary processes in green plant photosynthesis. Although the requirement for Mg^{2+} in photophosphorylation was noted by Arnon[27] in 1958, the stimulation of chloroplast fluorescence by Mg^{2+} was not reported until 1969 both by Homann[28] and Murata.[12] Murata observed that Mg^{2+} increased the fluorescence level of DCMU poisoned chloroplasts at room temperature. He also reported that at 77°K Mg^{2+} increased the emission observed at 685 nm and 695 nm (both identified with PS II on the basis of emission spectra of subchloroplast particles) and decreased the emission at 735 nm (usually ascribed to PS I on the basis of subchloroplast emission). In addition, Murata noted both an increase of 10-20% in the light limited rate of the $H_2O \rightarrow$ DCIP and a 20% decrease in the rate of the Asc/$DCIPH_2 \rightarrow NADP^+$ reaction, when Mg^{2+} was present in the chloroplast samples. These results led him to propose that Mg^{2+} blocks transfer of excitation energy (spillover) from PS II to PS I. Mohanty et al.[29] noted Mg^{2+} stimulation of fluorescence in untreated and tris-washed chloroplasts, but not in PS II subchloroplast particles. They concluded that both photosystems were needed to observe Mg^{2+} induced fluorescence increases; however, it should be noted that other explanations are possible. Briantais, Vernotte and Moya[30] reported that Mg^{2+} changed the shape of the variable fluorescence induction curve as well as its magnitude. They also observed that Mg^{2+} stimulated the light limited rate of DCIP reduction (40%) and decreased the rate of the ascorbate/$DCIPH_2$ to

methyl viologen reaction (35%). These authors also concluded that the primary role of Mg^{2+} was to block spillover from PS II to PS I. Avron and Ben-Hayyim[31] observed that Mg^{2+} increased the light limited rate of ferricyanide reduction by 75% and decreased the rate of Asc/DCIPH_2 → $NADP^+$ or methyl viologen by about 20%. They, like the other authors cited above, explained their results on the basis of Mg^{2+} blockage of spillover.

Sun and Sauer[16] reported that Mg^{2+} increased the rate of DCIP reduction by about 60%. They additionally reported[18] that enhancement in the $H_2O \rightarrow NADP^+$ reaction required the presence of Mg^{2+} or other divalent cations such as Mn^{2+}. They suggested that in addition to Murata's explanation for the role of Mg^{2+}, one must also consider an alternative model in which spillover of short wavelength quanta from PS I to PS II was promoted by the presence of Mg^{2+}. These two alternative formulations are summarized in Table I.1. The requirement for Mg^{2+} for enhancement in the $H_2O \rightarrow NADP^+$ reaction was challenged by Sane and Park[32] and McSwain and Arnon[19] but was strongly supported by the experiments of Sinclair[33] and Marsho and Kok.[14] On the basis of their studies of the intensity dependence of oxygen evolution, Marsho and Kok[14] also suggested that in addition to a role for Mg^{2+} involving changes in distribution of quanta between PS II and PS I, Mg^{2+} also increases energy transfer among PS II PSUs.

Using an amplitude-phase shift technique to measure the extent of electron flux through P700 and $NADP^+$, Rurianski et al.[34] concluded that Mg^{2+} decreased the electron flux through P700 while it increased the rate of $NADP^+$ reduction. They suggested that electron flow through

Table I.1.* Alternative Models for the Role of Divalent Cations in Enabling Red-far-red Enhancement for the $H_2O \rightarrow NADP$ Reaction by Broken Chloroplasts

Model A	Model B
1. Excitation transfer between Pigment System I occurs in the absence of divalent cations; not in their presence	1. Excitation transfer between Pigment System I and Pigment System II occurs in the presence of divalent cations; not in their absence
2. Intrinsic absorption of Pigment System II is greater than that of Pigment System I in the region 620 to 680 nm	2. Intrinsic absorption of Pigment System II is equal to that of Pigment System I in the region from 620 to 680 nm
Both models:	
3. Intrinsic absorption of Pigment System I is greater than that of Pigment System II at wavelengths longer than 690 nm	
4. Excitation transfer, when it is allowed, will occur predominantly in the direction which will enhance the activation of the reaction center that would otherwise be rate limiting	

* From reference 18.

P 700 was not linked to $NADP^+$ reduction and that Mg^{2+} altered the excitation distribution between P 700 and the reaction center associated with $NADP^+$ reduction.

Marsho and Kok[14] recently reported that Mg^{2+} alters the kinetics of P 700 oxidation by far red illumination for samples which had previously been illuminated by weak red light. These results seemed to indicate a control role for Mg^{2+} on the electron transfer chain between PS II and PS I. In addition to Mg^{2+} inhibition of the P 700

absorbance change, Mg^{2+} has also been reported by Gross and Libbey[35] to inhibit the 515 nm absorbance change, which is often associated with ionic gradients across the chloroplast membrane.

All of the above studies have been carried out on chloroplasts from higher plants (spinach, peas, lettuce, maize, wheat, and oats). Recently, however, Forti and Jennings[20] reported on Mg^{2+} effects on primary photosynthetic processes in Euglena. Since they observed Mg^{2+} stimulated fluorescence increases but no effect of Mg^{2+} on PS I reactions, they concluded that Mg^{2+} did not influence PS II-PS I spillover, but rather affected PS II only.

Studies have also been made on the relationship of these Mg^{2+} effects to cation induced changes in chloroplast structure.[36,37] This topic will be discussed in more detail in a subsequent chapter. The relationship of monovalent cation effects to divalent cation effects has also been an active area of recent research,[38,39] but this topic will not be treated further in this thesis.

It should be clear from the preceding discussion that the nature of the Mg^{2+} interaction with the primary processes of photosynthesis is exceedingly complex. The goal of the research discussed in this thesis was to define the sites of Mg^{2+} interaction with the primary photosynthetic processes and to provide an explanation for the mechanisms involved in these interactions. Specifically, we have examined Mg^{2+} effects on chloroplast fluorescence, the rates of electron transport, and the steady-state redox level of electron transport components as measured by the P 700 optical absorbance change.

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Chapter II

MAGNESIUM ION EFFECTS ON THE EFFICIENCY OF PHOTOSYNTHETIC ENERGY TRANSFER

Introduction

Fluorescence Studies: Room temperature fluorescence from chloroplasts shows emission characteristic of chlorophyll a (λ_{max} = 685 nm), regardless of whether the chlorophyll a is excited directly or receives its excitation via energy transfer from a light harvesting accessory pigment such as chlorophyll b, carotenoids or phycoerythrin.[1] Action spectra and studies on subchloroplast particles indicate that this room temperature emission can be associated almost completely with PS II.[2] (At liquid nitrogen temperature one observes peaks in the chloroplast emission spectrum at 685 nm, 695 nm and 735 nm.[3] On the basis of emission spectra of sub-chloroplast particles, the 685 nm and 695 nm peaks are usually identified with PS II, while the 735 nm peak is usually ascribed to PS I.)[3] Changes in room temperature fluorescence reflect changes in PS II excitation density.[4] These changes in excitation density result from changes in the processes responsible for the formation and relaxation of photo-excited PS II chlorophyll. The sensitivity of the fluorescence yield to changes in electron transport conditions[5] indicates that the competition among different molecular relaxation processes is affected by the efficiency of PS II electron transport. Thus changes in the level of actinic intensity, the presence of inhibitors, and the addition of electron acceptors alter the fluorescence yield.[5] By appropriate

choice of experimental parameters, we have been able to distinguish those Mg^{2+} effects on fluorescence which are independent of the state of electron transport from those effects which are sensitive to the efficiency of PS II electron flow.

Materials and Methods: Isolated broken chloroplasts were prepared from 14 to 21 day old Alaska pea plants (*Pisum sativum*) grown under the same conditions used by Sun and Sauer[6] in their studies on spinach chloroplasts. Pea leaves were picked after 4 to 6 hours' dark adaptation to provide chloroplasts with acceptable reproducibility of activity. Chloroplasts prepared from dark adapted pea leaves have been reported[7] to contain less Mg^{2+} than chloroplasts prepared from light adapted leaves. After removing the stems, the pea leaves were washed with distilled water and ground in 0.45 M sucrose, 0.1 M HEPES, pH 7.6, for 15 to 20 seconds in a Waring blendor equipped with a micro attachment. The leaf homogenate was filtered through 8 layers of Miracloth (Chicopee Mills, New York, N. Y.). The suspension was centrifuged at 200 x g for 1 minute to remove cell debris, and the supernatant was centrifuged at 1500 x g for 10 minutes. The pellet was resuspended in 2 ml of 0.45 M sucrose, 0.05 M HEPES, pH 7.6. For fluorescence measurements this suspension was diluted with the same medium to give a final absorbance (1 cm path) of about 0.3 at 436 nm.

Relative fluorescence intensities were measured using a modification of the method of Park *et al.*[2] A Cary 14 spectrophotometer equipped with a linear (% T) slidewire and a Model 1462 Scattered Transmission Accessory, modified for side illumination as described by Sauer and Biggins,[8] was operated in the reference mode so that the photomultiplier tube responded only to the modulated signal

arising from the sample cell. Using this technique, first introduced by Duysens and Sweers,[9] one can record the effect of an unmodulated actinic beam (side illumination) on the fluorescence signal excited by the low level modulated measuring beam without interference from emission that is induced by the unmodulated actinic source. Blue actinic illumination was provided by a Bausch and Lomb Hi Intensity Monochromator (bandwidth 12 nm) equipped with a 45 watt coil filament quartz iodide source. The photomultiplier (EMI 9558 QB) was protected from stray excitation light by a Schott RG 665 filter and an Optical Industries interference filter (bandwidth 10 nm, maximum transmittance 50% at 687 nm). The source for the modulated measuring beam was a Cary High Intensity Light Source operated at 120 volts. The Cary 14 monochromator had a bandwidth of 3.8 nm at 480 nm and 4.5 nm at 430 nm. A schematic representation of the experimental arrangement is shown in Figure II.1. Light intensities were measured using a Hewlett Packard Radiant Flux Meter #8330A and Probe #8334A or with an El Segundo Solar Cell and Keithley microvoltmeter. Both intensity measuring devices were calibrated against an Eppley thermopile and a NBS standard lamp. Unless otherwise stated, actinic illumination intensities were 55 $\mu w/cm^2$ at 480 nm and 38 $\mu w/cm^2$ at 430 nm. The measuring beam intensity at both 480 and 430 nm was determined to be less than 1 $\mu w/cm^2$.

To obtain a steady baseline for measurement of background fluorescence, previously dark adapted samples were inserted in the sample chamber, where the samples were exposed only to the weak measuring beam, and allowed to equilibrate for about 3 minutes.

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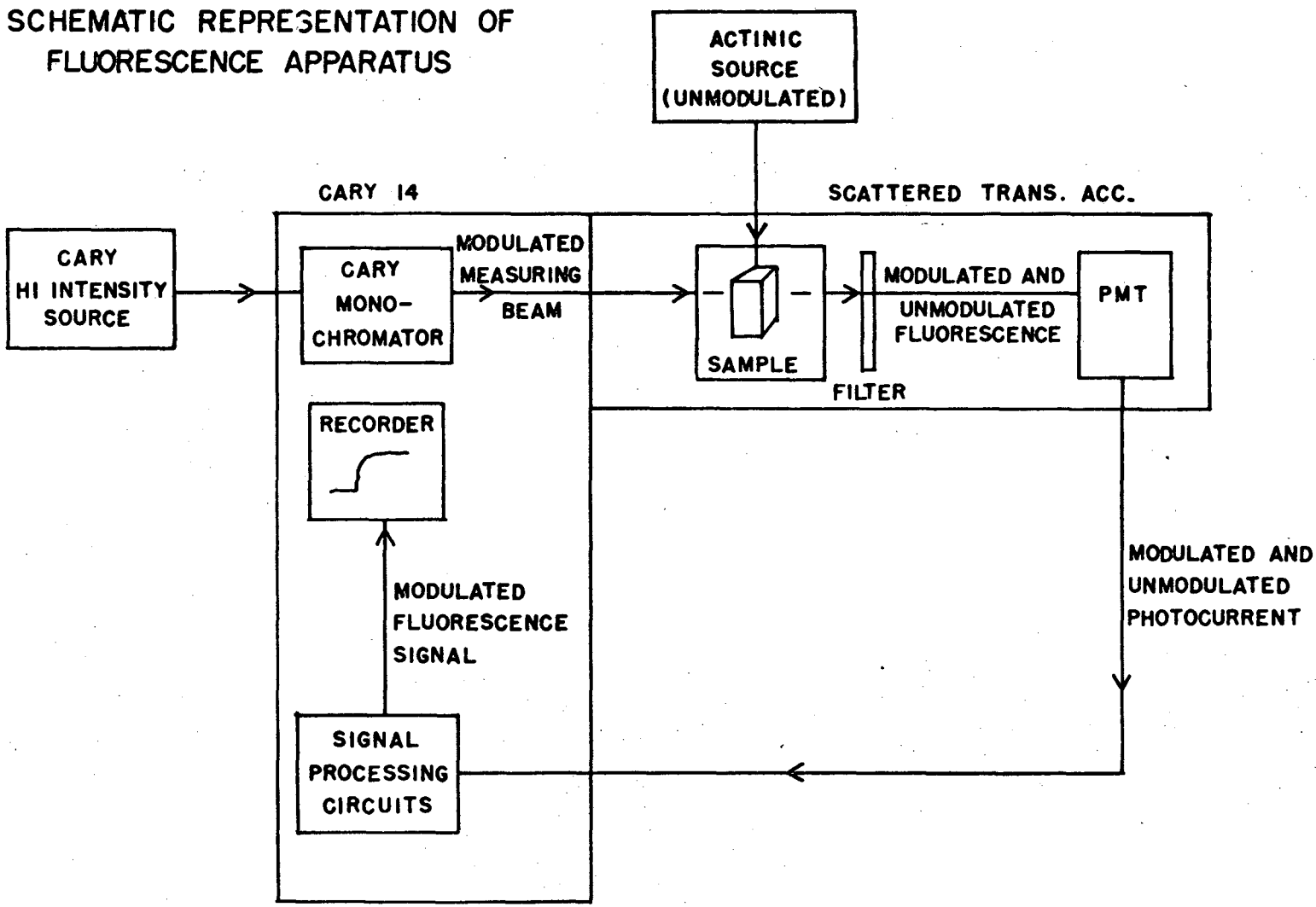

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Figure II.1. Schematic representation of fluorescence apparatus. Details as described in text.

Unless otherwise stated, all values reported for total and variable fluorescence levels are steady-state values obtained after 90 to 120 seconds of actinic illumination.

$NADP^+$, DCMU, plastocyanin, sucrose, DCIP and sodium ascorbate were obtained as described previously.[6] $MgCl_2$ was obtained from Mallinckrodt, ferredoxin from Sigma, and HEPES from Calbiochem.

Results: Figure II.2a shows that the modulated fluorescence intensity of a chloroplast sample can be increased over its normal background or dead fluorescence level if the sample is simultaneously illuminated by an unchopped actinic source. In a given experiment, the intensity of the modulated measuring excitation source remains constant. Consequently, changes in modulated fluorescence intensity correspond to changes in the relative efficiency of chloroplast fluorescence.

The increase in modulated fluorescence intensity caused by the actinic illumination is known as variable or live fluorescence.

Variable (F_V) and background (F_B) fluorescence refer strictly to the experimentally derived quantities shown in Figure II.2. As explained in detail in the Discussion section, live and dead fluorescence are theoretically defined quantities which refer to the origin of chloroplast fluorescence. Dead fluorescence represents emission which is independent of the condition of RC II, whereas live fluorescence competes with PS II photochemistry as a relaxation pathway for excited chlorophyll. For this reason, live fluorescence is sensitive to the ability of RC II to carry electron transfer reactions. If the modulated measuring beam is sufficiently weak and does not alter the

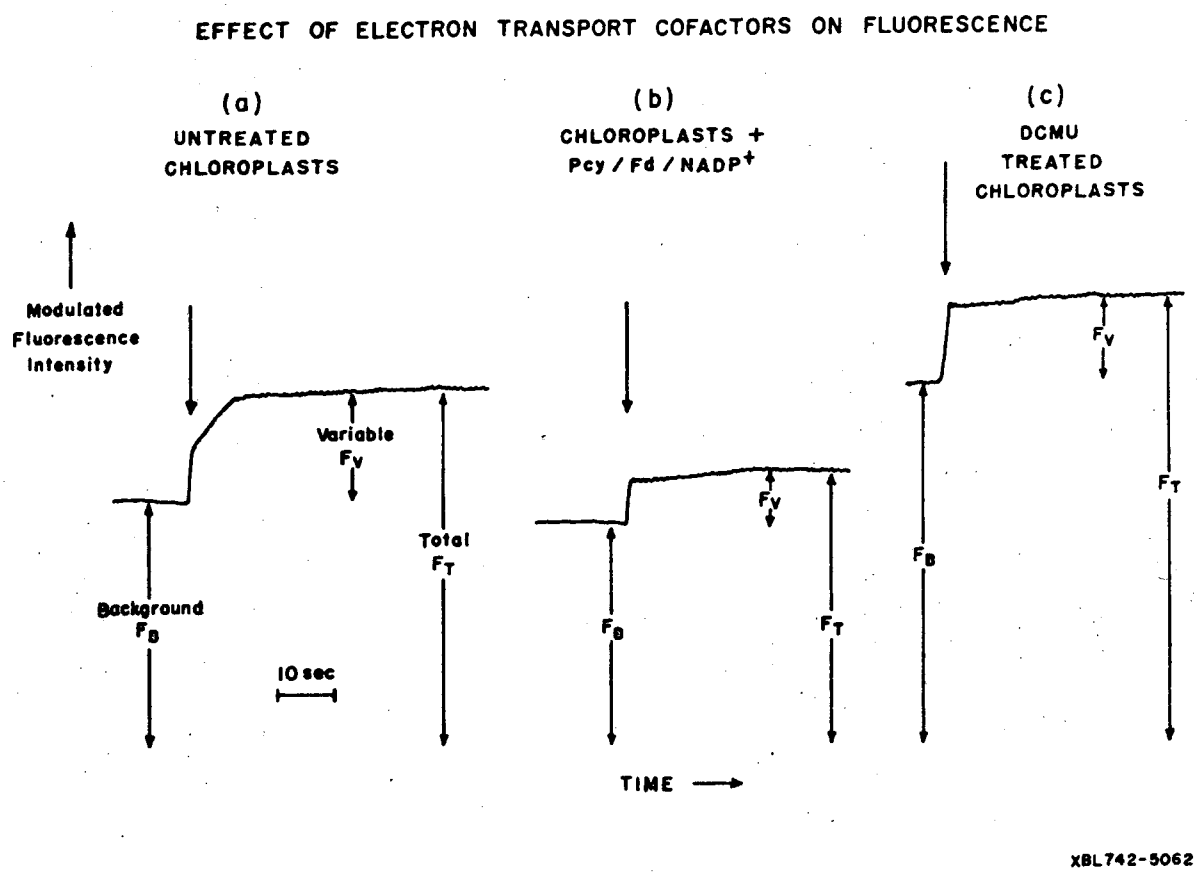

Figure II.2a. Fluorescence of untreated chloroplasts. Excitation wavelength, 480 nm; excitation intensity, 55 $\mu w/cm^2$; instrument time constant, 300 msec; other experimental details described in text. ↓ indicates start of actinic illumination. Variable fluorescence (F_V) is computed as the difference between the total fluorescence (F_T) and the background fluorescence (F_B).

Figure II.2b. Fluorescence of chloroplasts containing Pcy/Fd/$NADP^+$. Conditions as in Figure II.2a except that sample also contained the following additions: Pcy, saturating amount; Fd, 40 μg/ml; $NADP^+$, 0.6 mM.

Figure II.2c. Fluorescence of DCMU-treated chloroplasts. Conditions as in Figure II.2a except that sample also contained 0.01 mM DCMU.

fraction of functional reaction centers (open traps) then background and dead fluorescence are equivalent, as are variable and live fluorescence. For chloroplasts without DCMU, these conditions are often approximately if not exactly satisfied; hence in these studies we can often approximate dead fluorescence by background fluorescence and live fluorescence by variable fluorescence. (However, see discussion which follows below.) For DCMU poisoned chloroplasts we must use indirect techniques to estimate the fraction of the total fluorescence that is live in origin.

Duysens and Sweers[9] compared the effects of continuous illumination with light 1 (primarily absorbed by PS I) and light 2 (primarily absorbed by PS II) on the modulated live fluorescence yield of algae and chloroplasts. They observed that illumination with light 2 caused an increase in live fluorescence yield over the background level. When continuous illumination by light 2 was replaced by light 1 there was a decrease in the modulated live fluorescence yield. In the presence of DCMU, both light 1 and light 2 illumination caused increases in modulated fluorescence yield. The antagonistic effects of light 1 and light 2 observed in unpoisoned samples led Duysens and Sweers to suggest that the yield of chloroplast (live) fluorescence was controlled by the redox state of an electron carrier located on the electron transport chain between RC II and RC I. They proposed that this carrier, known as Q, quenched fluorescence in the oxidized state but did not quench fluorescence in the reduced (Q^- or QH) state. Duysens and Sweers[9] suggested that the quenching properties of Q indicated that it was closely associated with the PS II trap and that the oxidized form

of the quencher might be the primary electron acceptor of PS II. These authors also postulated that DCMU blocked electron transport from QH to secondary electron acceptors. The explanation of changes in chloroplast fluorescence yield in terms of the redox state of the PS II primary acceptor has proven successful in interpreting the effects of many electron transport cofactors on chloroplast fluorescence. For example, in Figure II.2b we note a decrease in live fluorescence yield of chloroplasts containing Pcy/Fd/$NADP^+$ relative to untreated chloroplasts (Fig. II2a). In the presence of this electron acceptor, Q should be more oxidized than in the untreated sample which lacks this exogenous acceptor. Thus we expect and observe a lower yield of fluorescence in chloroplasts containing Pcy/Fd/$NADP^+$ relative to untreated samples. In Figure II.2c we see an increase in total fluorescence yield of DCMU poisoned chloroplasts relative to the untreated samples. Since DCMU is postulated[9] to block electron flow from QH to secondary acceptos, we expect the primary acceptor to be predominantly reduced in the presence of this inhibitor. We therefore expect to observe the result shown in Figure II.2c: an increase in fluorescence in DCMU poisoned samples relative to untreated samples.

In our judgment, the most likely reason that the redox state of Q is an indicator of live fluorescence yield is that under most conditions the ability of the PS II trap to do photochemistry apparently is determined by the ability of the primary acceptor to accept electrons from the reaction center chlorophyll. Thus, when the acceptor is primarily reduced, observed under high actinic intensities and in

the presence of DCMU, the PS II trap cannot use excitation to transfer an electron to an (already reduced) acceptor so the excitation is wasted as fluorescence. When Q is primarily oxidized, observed in the presence of exogenous acceptors and at low actinic intensities, most of the excitation is used to transfer electrons from the trap to the primary acceptor; hence, not much excitation is available to be wasted as fluorescence. Under certain conditions such as low temperature (77°K) or very high time resolution, it is found that the fluorescence yield does not follow the redox state of the primary acceptor (measured as C 550).[10] Butler[10] has proposed that under these special conditions the fluorescence yield is influenced by the concentration of the primary PS II donor. (Butler[10] assumes that under most normal conditions the oxidized form of the donor is rapidly reduced and does not reach sufficient concentrations to exert an influence on fluorescence.) The results of Butler suggest to us that it is probably more useful to analyze live fluorescence in terms of the ability of the PS II trap to carry out electron transfer than solely in terms of the redox level of the primary acceptor (see Discussion).

The small decrease (10%) in the background fluorescence level between an untreated sample (no added acceptor) and the sample containing Pcy/Fd/$NADP^+$ probably indicates that in untreated chloroplasts the measuring beam causes a change in the redox state of RC II. Assuming that the measuring beam does not induce any live fluorescence when an acceptor is present, we conclude that the background fluorescence of untreated samples measured under these conditions is composed of about 90% dead fluorescence and 10% live fluorescence.

Figure II.3 shows the effect of added $MgCl_2$ on the levels of variable, background and total fluorescence of an untreated sample. Addition of $MgCl_2$ causes an increase of 30% in background fluorescence (curve a) saturating at about 0.5 mM $MgCl_2$. This Mg^{2+} concentration dependence is similar to the Mg^{2+} concentration profile for the inhibition of the 515 nm absorbance change reported by Gross and Libbey.[11] By contrast, the Mg^{2+} induced increase in variable fluorescence is about 250% and is maximal at 2.5 mM $MgCl_2$. The Mg^{2+} concentration dependence of variable fluorescence agrees with the data of Rurainski et al.[12] Recently Wydryzski, Gross and Govindjee[13] have also reported the existence of different Mg^{2+} concentration dependences for background and variable fluorescence. Using a different measurement technique, Homann,[14] Mohanty,[15] and Briantais et al.[16] also reported that Mg^{2+} stimulates larger increases in variable fluorescence than in background fluorescence. The existence of different Mg^{2+} concentration profiles for background and variable fluorescence indicates that the Mg^{2+} induced increase in background fluorescence does not result merely from the slight amount of live fluorescence which may be included in the measurement of background fluorescence.

Since the total modulated emission (background + variable) is 50-70% variable and the effect of Mg^{2+} is tenfold greater on the variable component, the Mg^{2+} concentration dependence of total emission (curve c) is similar to that observed for variable emission alone (curve b). Fluorescence studies with $MgSO_4$ showed that this salt was similar in effectiveness to $MgCl_2$ in stimulating variable and

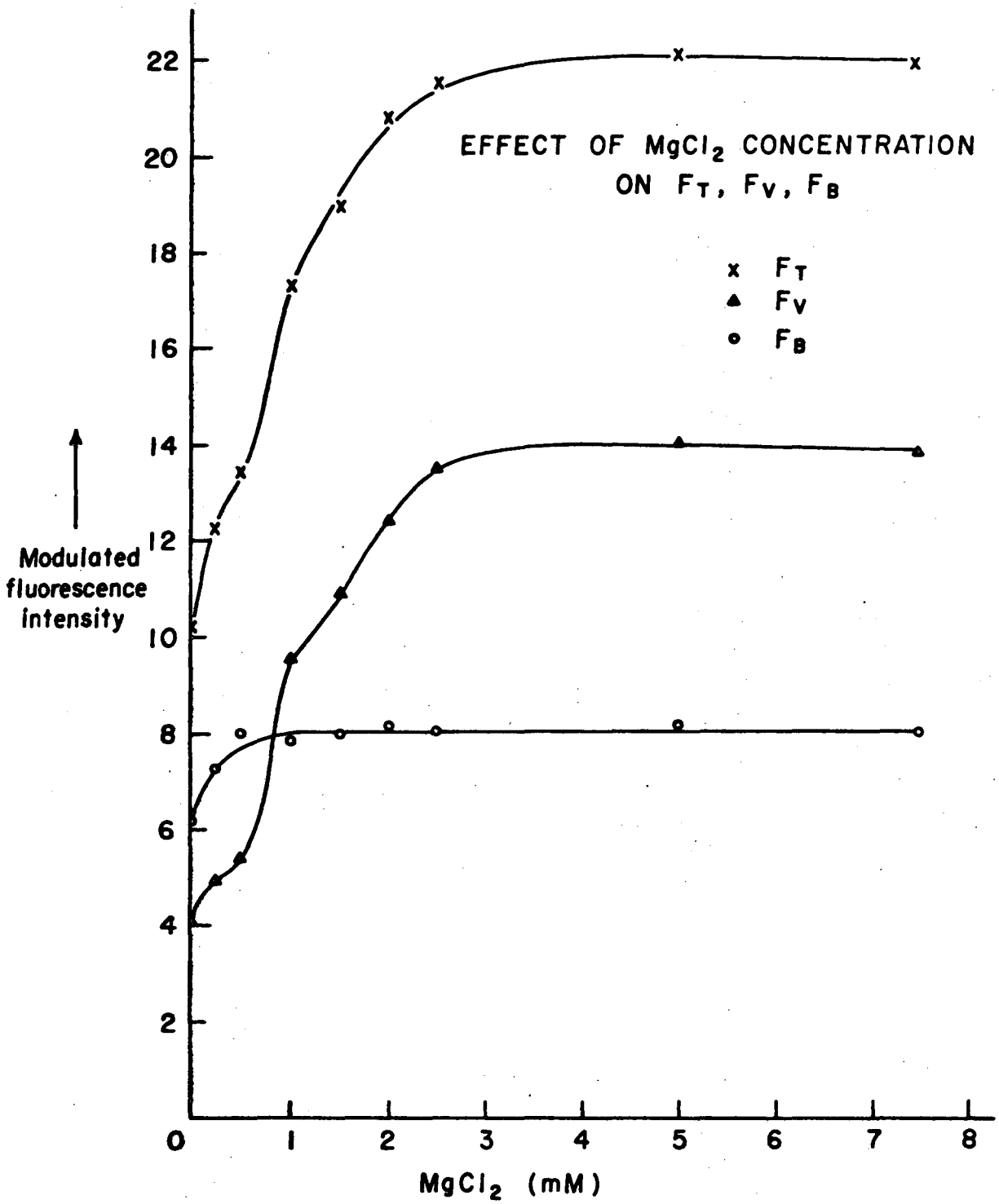

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Figure II.3. Effect of Mg^{2+} concentration on background, variable and total fluorescence levels in untreated chloroplasts. Other conditions as in Figure II.2a. F_V computed as described for Figure II.2a.

background fluorescence. Preliminary experiments indicated that the aliphatic amine spermine was capable of replacing Mg^{2+} in these fluorescence stimulation experiments. $MgCl_2$ stimulation of fluorescence was abolished upon addition of EDTA.

Figure II.4a shows the effect of $MgCl_2$ concentration on the kinetics of variable fluorescence in untreated chloroplasts. It shows that the addition of 0.5 mm $MgCl_2$ to untreated chloroplasts increases the level of both components of fluorescence over the value observed for chloroplasts without Mg^{2+}. However, as shown in Figure II.4a, samples with no Mg^{2+} or 0.5 mm Mg^{2+} reach the steady-state level of variable fluorescence in less than 15 seconds of actinic illumination. In contrast, the sample with 5 mm Mg^{2+} has reached only about 5/6 of its steady-state value after 15 seconds of actinic illumination. After 30 seconds of actinic illumination, this sample has achieved about 11/12 of its steady-state value. As seen in Figure II.4b, this long induction period in Mg^{2+} treated samples is seen only upon the first illumination of dark adapted untreated chloroplasts. Upon the second illumination, the sample with 5 mM Mg^{2+} reaches the maximum variable fluorescence level in about the same time (less than 12 seconds) as the sample with no $MgCl_2$. We also observe that the F_B level after illumination is greater than the F_B level before illumination.

Figure II.5 shows the effect of weak ($\leq$ 40 $\mu w/cm^2$) actinic intensity on the variable fluorescence level of chloroplasts which contain the electron acceptor DCIP. Under these experimental conditions, we observe that F_V is directly proportional to I_{act} in

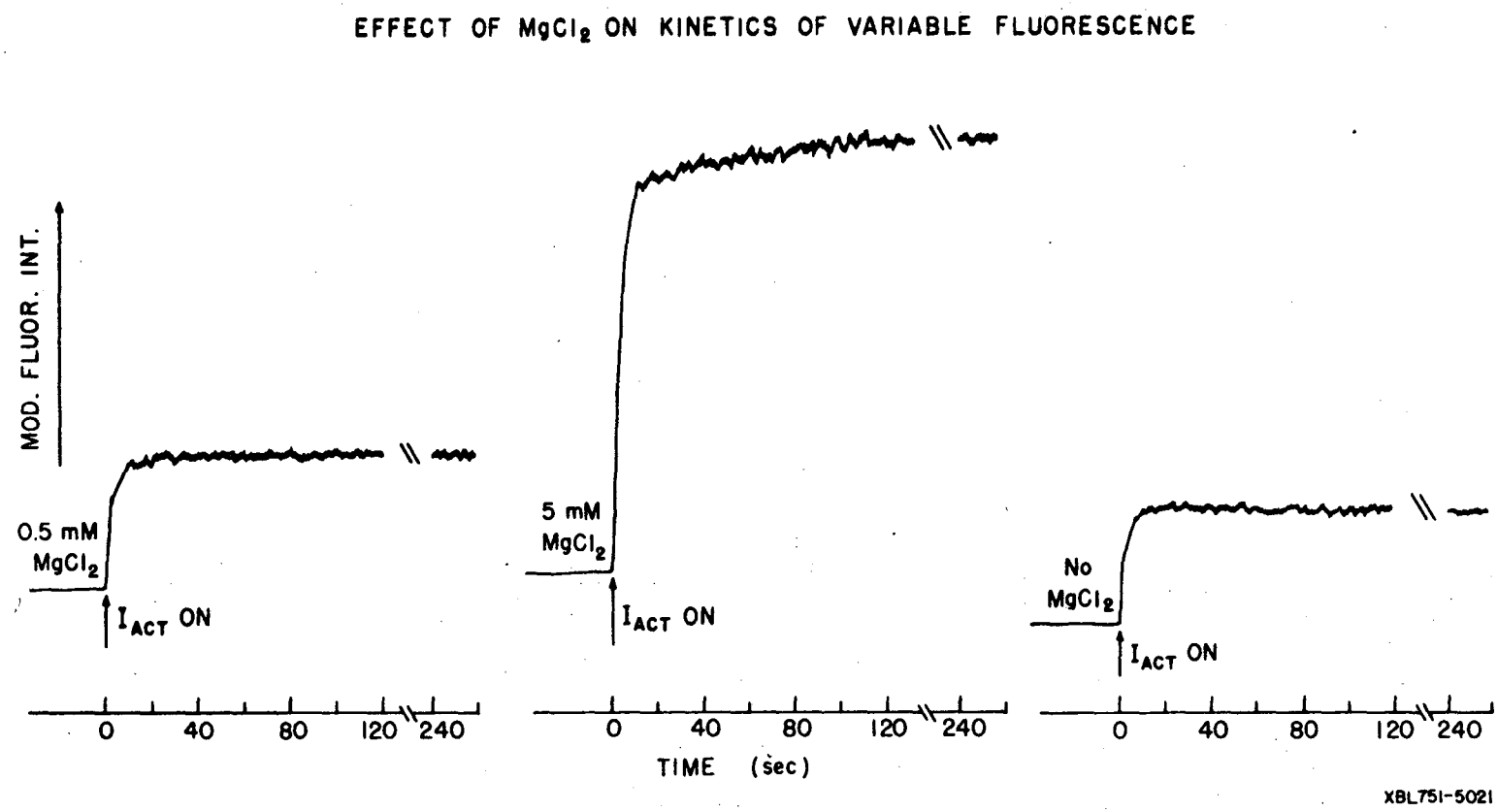

Figure II.4a. Effect of $MgCl_2$ on induction kinetics of variable fluorescence. Other conditions as in Figure II.2a.

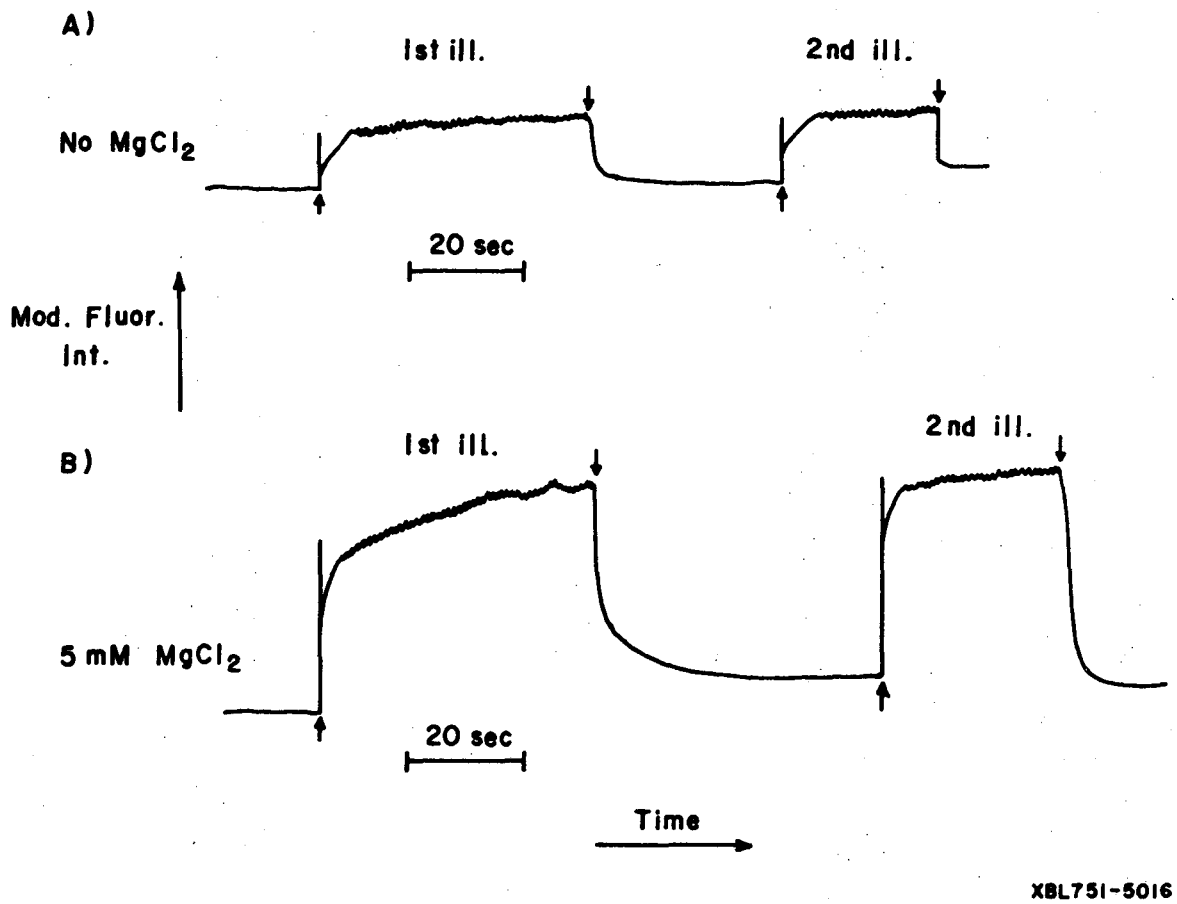

Figure II.4b. Effect of $MgCl_2$ and illumination sequence on kinetics of variable fluorescence. A) No $MgCl_2$; B) 5 mM $MgCl_2$. ↑ indicates start of actinic illumination; ↓ indicates end of actinic illumination. Other experimental conditions and procedures as in Figure II.2a.

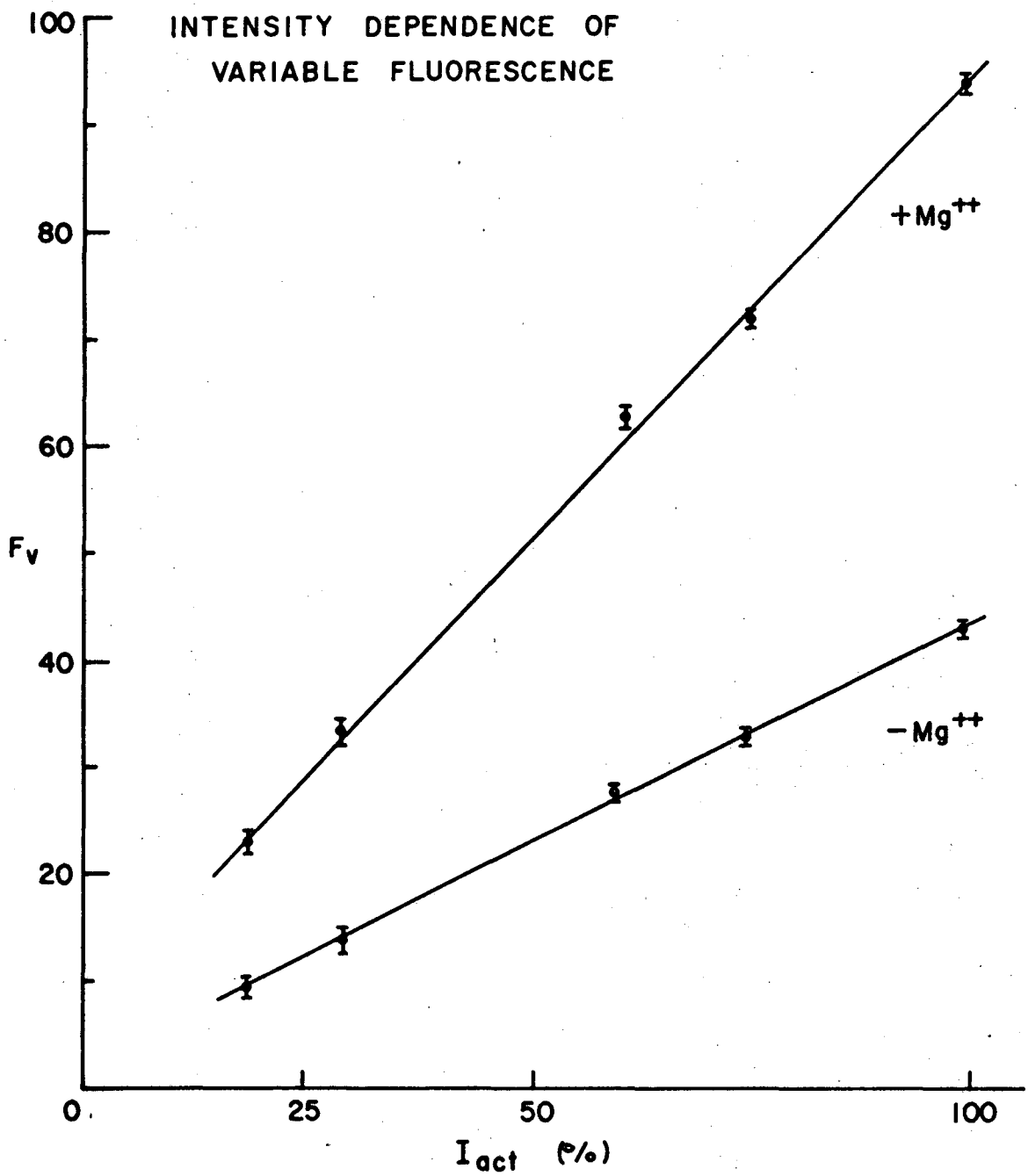

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Figure II.5. Effect of Mg^{2+} on intensity dependence of variable (live) fluorescence in chloroplasts containing DCIP. DCIP concentration, 0.02 mM; I_{act} value of 100% represents an actinic intensity of 40 $\mu w/cm^2$. Other experimental conditions as in Figure II.2a. F_V is computed as in Figure II.2a.

chloroplast samples with or without Mg^{2+} The slope of the F_V versus I_{act} plot is related to the fluorescence yield (see Discussion) and is significantly altered by the presence of Mg^{2+} In particular, the slope of the F_V versus I_{act} plot for Mg^{2+} containing samples is twice the value obtained for samples without Mg^{2+}. The approximate linearity of the F_V versus I_{act} plot in the presence and absence of Mg^{2+} indicates that at these low intensities the effectiveness of actinic intensity in stimulating variable fluorescence (F_V/I_{act}) is essentially independent of actinic intensity.

Using higher actinic intensities ($\leq 65\ \mu w/cm^2$), we have examined the intensity dependence of the quantity F_V/I_{act} in untreated chloroplasts. Figure II.6 shows that F_V/I_{act} is approximately independent of I_{act} for samples without Mg^{2+}, but is sharply, almost hyperbolically dependent on actinic intensity for Mg^{2+} containing samples. Although there is some degree of variation in the precise shape of the F_V/I_{act} plot for different chloroplast preparations, we always observe in this intensity region that F_V/I_{act} changes sharply with I_{act} for Mg^{2+} containing samples. These results indicate that Mg^{2+} treated samples approach light saturation of variable fluorescence at much lower actinic intensities than do samples without Mg^{2+}. This conclusion is supported by the observation made by Mohanty et al.[15] that addition of DCMU causes a smaller fluorescence increase for Mg^{2+} containing samples than for samples without Mg^{2+}. The addition of DCMU to a chloroplast sample results in a state with the maximum (live) fluorescence yield. We therefore conclude that before the addition of the inhibitor samples with Mg^{2+} are closer to saturation than are preparations without Mg^{2+}.

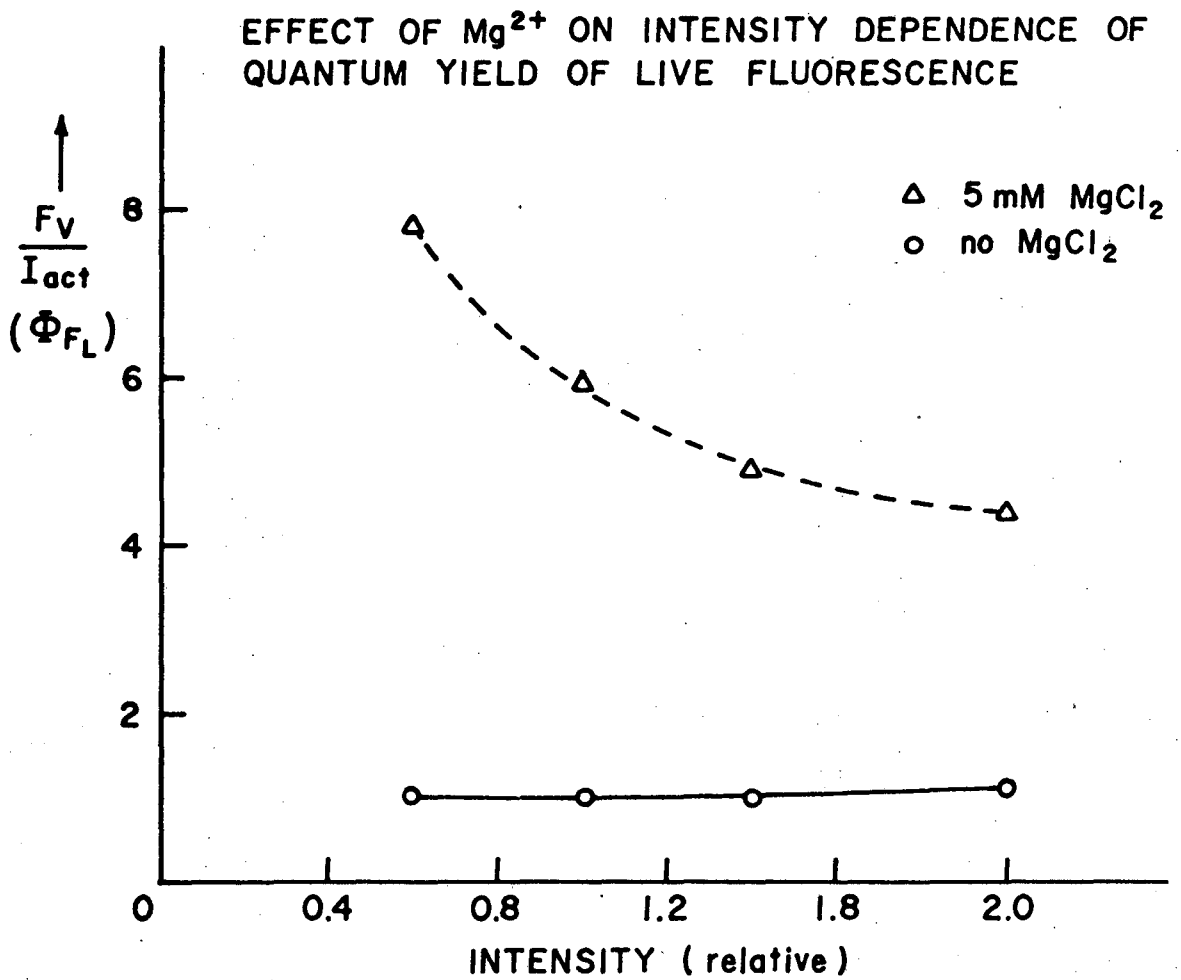

Figure II.6. Effect of Mg^{2+} on intensity dependence of F_V/I_{act} in untreated chloroplasts. Relative intensity of 2.0 represents actinic intensity of 65 $\mu w/cm^2$. Other experimental conditions as in Figure II.2a. F_V is computed as described for Figure II.2a.

Table II.1 shows that chloroplasts with added electron acceptors, like untreated chloroplasts, show much greater Mg^{2+} stimulation of variable fluorescence than of background fluorescence. Compared to untreated chloroplasts, chloroplasts with added electron acceptors show smaller Mg^{2+} induced changes in background, variable and total fluorescence.

The addition of the inhibitor DCMU causes a 20-30% increase in the level of total fluorescence (Fig. II.2c). Table II.1 confirms previous reports that addition of Mg^{2+} to DCMU poisoned chloroplasts doubles the amount of total fluorescence. Figure II.7 confirms the report of Murata[17] that Mg^{2+} stimulation of total fluorescence in DCMU poisoned samples saturates at a Mg^{2+} concentration of 2.5 mM. As shown in Table II.1, one observes a doubling of total fluorescence yield when Mg^{2+} is added to chloroplasts which contain sodium dithionite. This result confirms the earlier report of Homann.[14]

In order to test whether Mg^{2+} action occurs by altering spillover between PS II and PS I, we compared Mg^{2+} effects on emission excited at 480 nm (primarily PS II) to 430 nm (preferentially PS I). The quantity R tabulated in Table II.2 for background, variable and total emission compares the relative effectiveness of Mg^{2+} in stimulating fluorescence increases activated at 480 nm to 430 nm. No significant wavelength effect on Mg^{2+} induced background fluorescence was observed for sample with or without electron acceptors. Those samples without added acceptors also show little effect of actinic wavelength for Mg^{2+} stimulated variable fluorescence (R_V = 1.1). However, with added acceptors a substantially greater Mg^{2+} effect on 480 than 430 nm

Table II.1

Effect of Electron Transport Cofactors on Mg^{2+} Stimulated Fluorescence Increases

Experimental conditions: excitation wavelength, 480 nm; excitation intensity, 55 μw-cm^{-2}; $MgCl_2$, 5 mM; Fd, 34 μg/ml; $NADP^+$, 0.5 mM; dithionite and Pcy, saturating amount; DCMU, 50 μM; DCIP, 3 μM; conditions as described in Methods section. F_B, F_V, F_T as defined in Fig. II.2

	$F_B{}^{+Mg^{2+}}/F_B{}^{-Mg^{2+}}$	$F_V{}^{+Mg^{2+}}/F_V{}^{-Mg^{2+}}$	$F_T{}^{+Mg^{2+}}/F_T{}^{-Mg^{2+}}$
Untreated chloroplasts	1.29 ± 0.08	3.70 ± 0.25	2.24 ± 0.17
Chloroplasts + Fd/$NADP^+$	1.16 ± 0.05	3.05 ± 0.19	1.84 ± 0.05
Chloroplasts + Pcy/Fd/$NADP^+$	1.20 ± 0.04	2.95 ± 0.16	1.46 ± 0.07
Chloroplasts + DCIP	1.14 ± 0.03	3.09 ± 0.19	1.64 ± 0.05
Chloroplasts + DCMU	--	--	2.11 ± 0.11
Chloroplasts + dithionite	--	--	2.1 ± 0.15

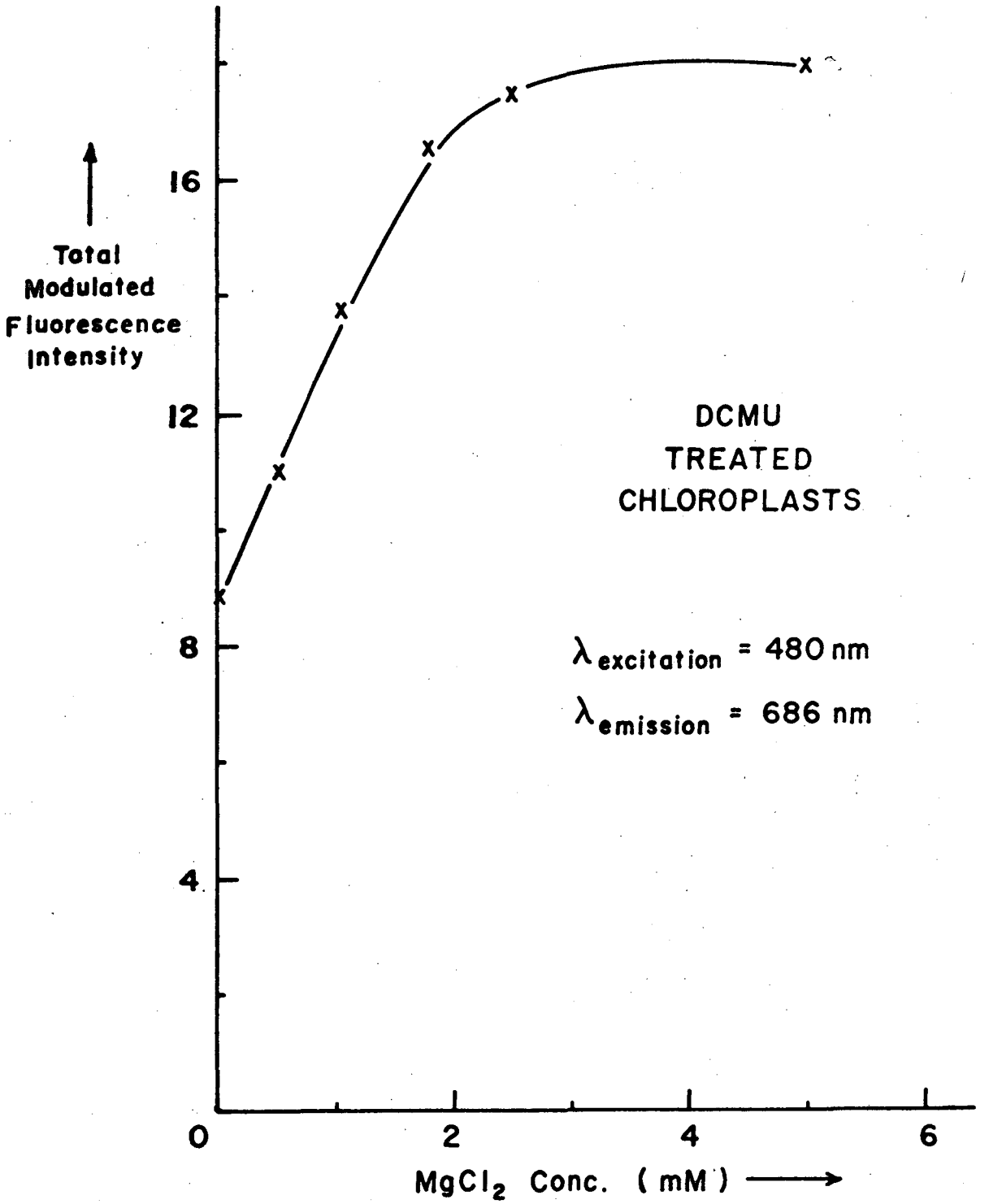

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Figure II.7. Effect of Mg^{2+} concentration on total fluorescence level of DCMU-treated chloroplasts. Other conditions as in Figure II.2c.

Table II.2

Effect of Actinic Wavelength on Mg^{2+} Stimulated Fluorescence Increases

Actinic excitation intensity at 430 nm, 38 μw-cm^{-2}; at 480 nm, 55 μw-cm^{-2}; other experimental conditions as in Table II.1. R is defined as follows:

$$R = \frac{F^{+Mg^{2+}}/F^{-Mg^{2+}} \text{ at 480 nm}}{F^{+Mg^{2+}}/F^{-Mg^{2+}} \text{ at 430 nm}} \quad \text{for } R_B\text{: } F=F_B\text{, for } R_V\text{: } F=F_V\text{, for } R_T\text{: } F=F_T$$

	R_B	R_V	R_T
Untreated chloroplasts	1.06 ± 0.05	1.12 ± 0.13	1.10 ± 0.07
Chloroplasts + Fd/NADP$^+$	1.02 ± 0.04	1.46 ± 0.11	1.23 ± 0.05
Chloroplasts + Fd/Pcy/NADP$^+$	1.05 ± 0.05	1.62 ± 0.13	1.12 ± 0.04
Chloroplasts + DCIP	1.07 ± 0.05	1.52 ± 0.11	1.26 ± 0.05
Chloroplasts + DCMU	--	--	1.10 ± 0.04

activated variable fluorescence is apparent. These results[14] do not appear to support Murata's proposal[17] that Mg^{2+} blocks spillover from PS II to PS I (see Discussion). As reported by Homann,[14] DCMU poisoned samples show only a slight wavelength effect on Mg^{2+} stimulated total fluorescence.

Discussion

Figure II.3 shows that Mg^{2+} stimulation of live (variable) fluorescence saturates at a higher Mg^{2+} concentration than does Mg^{2+} stimulation of dead (background) fluorescence. This result indicates that Mg^{2+} stimulation of live fluorescence has a different origin than Mg^{2+} stimulation of dead fluorescence. One possible interpretation is that there are separate sites of live and dead fluorescence (two site model). In this two site formulation, live and dead fluorescence are emitted by different groups of pigments, and the Mg^{2+} effects are independent of one another. An analysis of Mg^{2+} effects on fluorescence in terms of this model will be presented below. An alternative model involves a single fluorescing site. In the single site formulation both live and dead emission come from the same group of emitting pigments. An analysis of Mg^{2+} effects on fluorescence in terms of a single site model follows the discussion of results in terms of a two site model.

The interpretation of Mg^{2+} effects on live fluorescence for either of these models relies strongly on the intensity dependence of live (variable) fluorescence data shown in Figure II.5. The data shown in this figure are particularly useful, because knowledge of the intensity dependence of live fluorescence allows us to obtain

relative values for the relaxation processes involved in de-excitation of PS II live chlorophyll. Knowing these relative values, we can determine whether the previous explanation[15-17] of Mg^{2+} induced increases in live fluorescence in terms of inhibition of PS II→PS I spillover is tenable.

Two Site Model: According to this model (Fig. II.8), PS II pigments are arranged in two different types of emitting arrays. One array (group) of pigments (the live pigment array) both sensitizes and emits live fluorescence. Similarly, the other group of pigments, the dead pigment array, both sensitizes and emits dead fluorescence. According to this model, excitation absorbed by pigments in the live pigment array can be transferred to an open trap for PS II photochemistry or can be degraded via radiative (fluorescence) or non-radiative (thermal + spillover) pathways. Thus, fluorescence from the live pigment array competes with PS II photochemistry as a relaxation pathway for excitation absorbed by this group of pigments. In contrast, pigments in the dead pigment array are unable to transfer energy to the PS II trap. Consequently the fluorescence yield of the dead pigment array is independent of actinic intensity.

The Nature of the Live Fluorescence Site: If we consider the live pigment array to include the PS II trap in addition to the antenna pigments that sensitize live fluorescence, there are two alternative emitting sites for live fluorescence. According to one view, the fluorescent trap hypothesis (A), live fluorescence arises from excitation which is trapped and subsequently emitted directly by the PS II reaction center. An alternative viewpoint, the fluorescent array model (B),

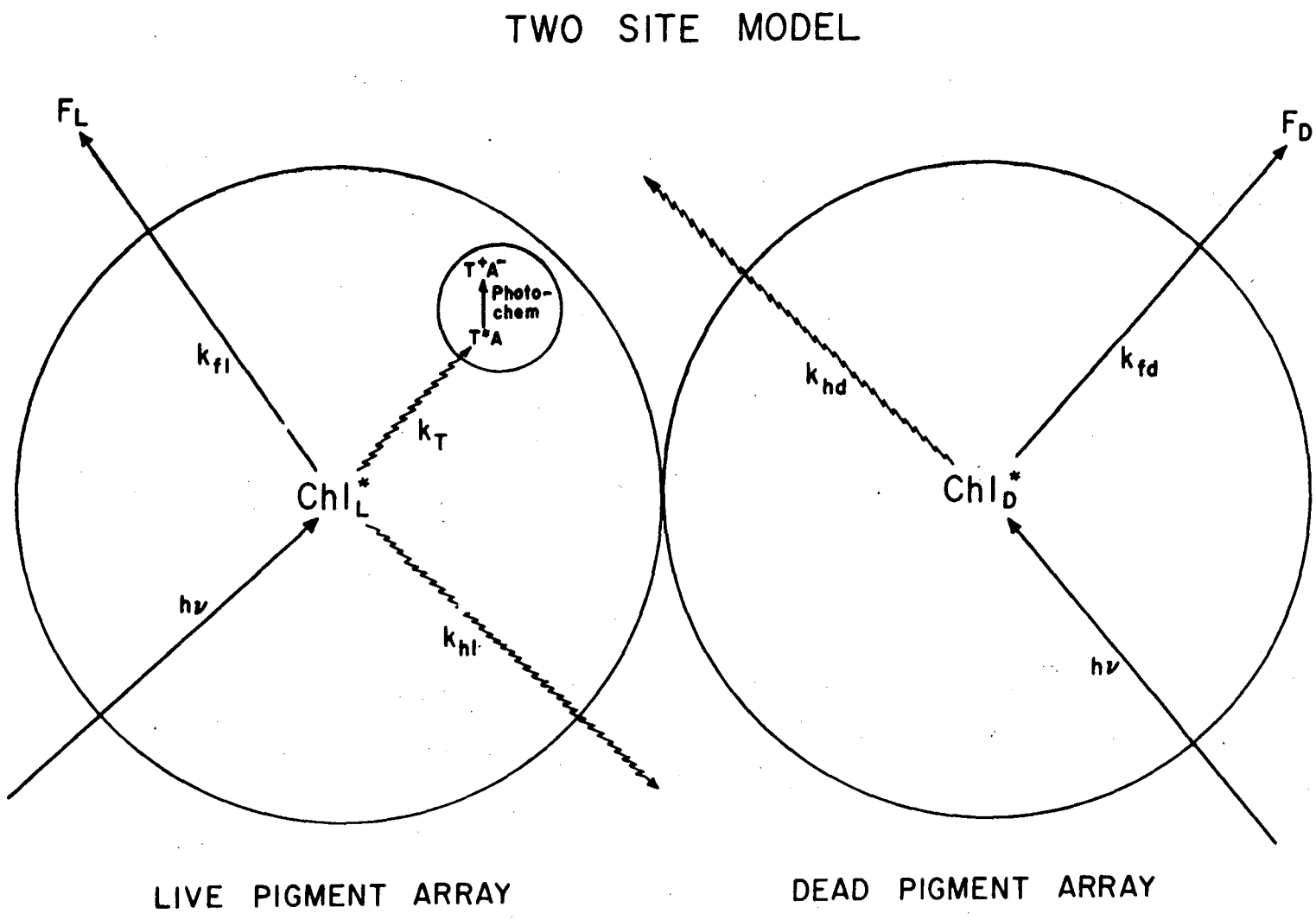

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Figure II.8. Two site model for chloroplast fluorescence. Details explained in text.

would be that a PS II reaction center chlorophyll which cannot carry out photochemistry (a closed trap) cannot act as energy sink or trap. In this case, the closed trap is no longer distinguishable from other molecules in the live pigment array. Consequently, in the fluorescent array model (B), live fluorescence is a property of the entire live pigment array and not the reaction center chlorophyll alone.

According to Lavorel and Joliot[18] the shape of the chloroplast fluorescence induction curve can sometimes be used to distinguish a fluorescent array model from a fluorescent trap model. These authors show that a sigmoidal induction curve is only compatible with a fluorescent array formulation,while an exponential induction curve cannot unambiguously distinguish a fluorescent array model (B) from a fluorescent trap model (A). The sigmoidal induction curves for normal chloroplasts reported by Lavorel and Joliot,[18] Delosme,[19] and Briantais *et al.*[16] indicate that the fluorescent array model presently provides a more likely explanation for the site of live fluorescence than does the fluorescent trap model. (The assumption of a live fluorescent array has been used implicitly by Mohanty *et al.*[15] and Briantais *et al.*[16] in their discussions of chloroplast fluorescence.) The processes involved in live fluorescence are summarized in the following equations:

		Rate
Excitation	$Chl_L + h\nu \rightarrow Chl_L^*$	$\sigma_L I_m$
Trapping & Photochemistry	$Chl_L^* + TA \xrightarrow[k_T]{} Chl_L + T^*A \rightarrow$ Photochem.	$k_T(TA)(Chl_L^*)$
Fluorescence	$Chl_L^* \xrightarrow[k_{fl}]{} Chl_L + h\nu'$	$k_{fl}(Chl_L^*)$
Thermal decay & spillover to PS I	$Chl_L^* \xrightarrow[k_{hl}]{} Chl_L$ + "heat"	$k_{hl}(Chl_L^*)$

where Chl_L = ground state pigment in live array

Chl_L^* = excited singlet state pigment in live array

TA = ground state of open reaction center

T*A = excited state of reaction center

TA^- = closed state of reaction center

k_T = rate constant for energy transfer from live excited pigment to reaction center

k_{hl} = rate constant for de-excitation of live pigments by thermal losses and spillover to PS I

k_{fl} = rate constant for de-excitation of excited live pigments by fluorescence

σ_L = effective absorption cross section (size) of PS II live pigment array

F_L = level of live fluorescence

I_m = intensity of modulated measuring beam

We can represent the level of live fluorescence in this model by the following equation:

$$F_L = \left(k_{fl}\ I_m\ \sigma_L\right) / \left(k_T\ (TA) + k_{hl} + k_{fl}\right) \qquad \text{(Eq. II.A)}$$

According to this model, changes in actinic intensity affect live fluorescence yield by altering the ratio of open to closed traps. In particular, increases in actinic intensity are assumed to decrease the magnitude of the term (TA) in equation II.A. Since Figure II.5 shows that live (variable) F_L (F_V) increases linearly with actinic intensity for samples with and without Mg^{2+}, we can conclude that in the intensity region under consideration (TA) α $1/I_{act}$ and k_T (TA) >> k_{fl} and k_{hl} for samples with or without Mg^{2+}. It is unlikely that the Mg^{2+} stimulated increases in live fluorescence arise from a Mg^{2+} induced decrease in the k_T (TA) term in equation II.A, since the data of Table II.1 and reference 14 show that DCMU and dithionite treated chloroplasts, which cannot carry out photochemistry (k_T (TA) $\approx$ 0), still show a doubling of total fluorescence upon addition of Mg^{2+}. Furthermore, chloroplasts with and without acceptors, which therefore contain different fractions of open and closed traps (different values of k_T (TA)), show similar large Mg^{2+} induced live fluorescence increases (Table II.1). Since k_{fl} is generally assumed to be constant, the most reasonable origin in this model for a Mg^{2+} induced increase in live fluorescence is a Mg^{2+} induced increase in σ_L. An increase in σ_L indicates an increase in the effective size (absorption cross section) of the PS II live antenna pigment array. A Mg^{2+} stimulated increase in spillover from PS I $\rightarrow$ PS II, previously proposed by Sun and Sauer[6] would correspond to an increase in σ_L. Mg^{2+} "activation" or "connection" of previously "inactive" or "disconnected" chlorophyll would also correspond to an increase in σ_L. The requirement that k_{hl}, which represents degradative losses by heat and spillover to PS I, is much less than

k_T (TA) is significant, because it indicates that a Mg^{2+} induced decrease in k_{hl} cannot account for the Mg^{2+} induced doubling of live fluorescence shown in Figure II.5. Previously Murata[17] and others[15,16] have proposed that Mg^{2+} stimulated fluorescence increases arise from Mg^{2+} induced blockage of spillover from PS II → PS I. However, the analysis presented above shows that this proposal is no longer tenable for a two site model.

Dead Fluorescence: In terms of our two site model, we can represent the processes involved in dead fluorescence by the following equations:*

Excitation	$Chl_D + h\nu \rightarrow Chl_D^*$	$\sigma_D I_m$
Thermal decay	$Chl_D^* \xrightarrow[k_{hd}]{} Chl_D + heat$	$k_{hd}(Chl_D^*)$
Fluorescence	$Chl_D^* \xrightarrow[k_{fd}]{} Chl_D + h\nu'$	$k_{fd}(Chl_D^*)$

We can therefore write the following equation for the level of dead fluorescence:

$$F_D = \left(k_{fd}\ \sigma_d I_m\right) / \left(k_{hd} + k_{fd}\right) \qquad \text{(Eq. II.B)}$$

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*We have also considered the possibility of sensitization of dead fluorescence via energy transfer from the live array:

$$Chl_L^* + Chl_D \rightarrow Chl_L + Chl_D^*$$

In a fluorescent array formulation of live fluorescence, Chl_L^* is sensitive to conditions that affect the fraction of open traps; hence, the above equation predicts that dead fluorescence should also be sensitive to the state of the trap. Since dead fluorescence is defined to be independent of the state of the trap, we have not included the above equation in our discussion of dead fluorescence.

where Chl_D = ground state pigment in dead array

Chl_D^* = excited singlet state of pigment in dead array

Chl_L^* = excited signlet state of pigment in live array

σ_D = effective absorption cross section (size) of dead pigment array

k_{hd} = rate constant for thermal (non-radiative) decay of excited dead pigment

k_{fd} = rate constant for fluorescence deactivation of excited dead pigment

F_D = level of dead fluorescence

I_m = intensity of measuring beam

According to this model, a Mg^{2+} stimulated increase in dead fluorescence could arise from a Mg^{2+} induced increase in k_{fd} or σ_D, or from a Mg^{2+} induced decrease in k_{hd}. Making the usual assumption that k_{fd} is a true constant of the system which is unaltered by the presence of Mg^{2+}, we can still account for a Mg^{2+} stimulated increase in dead fluorescence either by an increase in σ_D, corresponding to an increase in the effective size of the dead pigment array, or a decrease in the rate constant for non-radiative relaxation, k_{hd}. Since little is known about the detailed molecular properties of these dead pigments, it is not yet possible to determine which of these two terms is responsible for the observed Mg^{2+} induced increase in dead fluorescence.

Single Site Models: An alternative to the two site model discussed above is a single site model. In a single site model both live and dead fluorescence are emitted by the same group of emitting pigments. The simplest type of single site model involves the case where the same group of pigments sensitizes and emits both components of

fluorescence. As shown in the appendix it is not possible to reconcile the experimental data of Figure II.5 and Figure II.3 with this particular type of single site model.

Figure II.9 shows a representation of a different single site model in which there are three types of pigment arrays. In this view there are different groups of sensitizing pigments for live and dead fluorescence but a common group of emitting pigments for both components of fluorescence. The properties of these different groups of pigments will be discussed below.

1. Live Pigment Array: This group of pigments can transfer absorbed excitation to the PS II reaction center for photochemistry or degrade excitation via thermal degradation (including spillover) or transfer excitation to the fluorescent pigment array. (It is an implicit property of this model that pigments in the live array are non-fluorescent. This means that the rate constant of radiative decay of these pigments must be much less than the rate constants of competing relaxation processes. Consequently, the rate constant of radiative decay of these pigments will not be considered in the discussion below.) Energy transfer from molecules in the live pigment array to molecules in the fluorescent pigment array thus competes with PS II photochemistry as a relaxation pathway for excitation absorbed by the live group of pigments. Since the yield of PS II photochemistry depends on actinic intensity it is clear that the yield of energy transfer from the live pigment array to the fluorescent pigment array will also be intensity dependent. Hence the yield of live fluorescence will also be a function of the actinic intensity.

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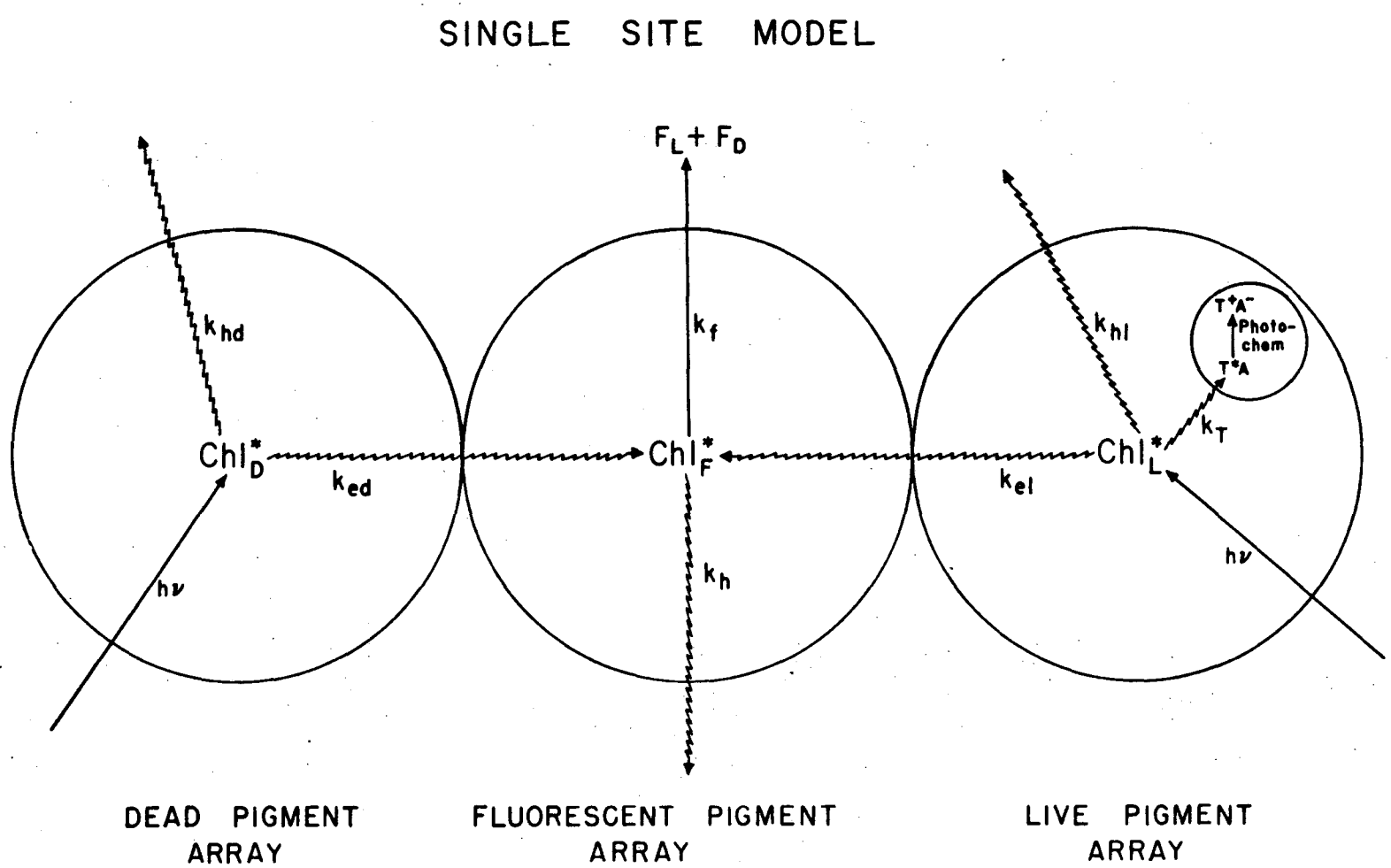

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Figure II.9. Single site model for chloroplast fluorescence. Details explained in text.

2. Dead Pigment Array: The pigments in the dead group are assumed to be functionally disconnected from the live array and PS II trap, but able to transfer excitation to the fluorescent pigment array. Since the ability of the dead pigments to sensitize fluorescence is independent of the state of PS II photochemistry, the yield of dead fluorescence is unaffected by actinic intensity. (As with the live group of pigments, pigments in the dead array are assumed to be non-fluorescent. Consequently, the rate of radiative decay of these pigments will not be included in further discussion.)

3. Fluorescent Pigment Array: This group of pigments, which probably represents only a small fraction of the total PS II chlorophyll, receives excitation from both the live and dead pigment arrays and is the site of emission of both live and dead fluorescence. Thus, according to this interpretation, there is only one group of emitting pigments which is sensitized via excitation transfer from two different groups of absorbing pigments.

Live Fluorescence: The processes which determine the level of live fluorescence involve the state of PS II photochemistry and the efficiency of energy transfer from the live pigment array to the fluorescent pigment array. These processes are summarized in the following equations: (As in the two site model, we have assumed that a closed trap is indistinguishable from other antenna pigments in the live array.)

		Rate
Excitation	$Chl_L + h\nu \rightarrow Chl_L^*$	$\sigma_L I_m$
Trapping & Photochemistry	$Chl_L^* + TA \xrightarrow[k_T]{} Chl_L + T^*A \rightarrow$ Photochem.	$k_T(TA)(Chl_L^*)$
Thermal decay & spillover to PS I	$Chl_L^* \xrightarrow[k_{hl}]{} Chl_L$ + "heat"	$k_{hl}(Chl_L^*)$
Energy transfer to fluorescent array	$Chl_L^* + Chl_F \xrightarrow[k_{el}]{} Chl_L + Chl_F^*$	$k_{el}(Chl_L^*)Chl_F)$
Fluorescence	$Chl_F^* \xrightarrow[k_f]{} Chl_F + h\nu'$	$k_f(Chl_F^*)$
Thermal relaxation of fluorescent array	$Chl_F \xrightarrow[k_h]{} Chl_F$ + "heat"	$k_h(Chl_F^*)$

where Chl_L = ground state pigment in live array

Chl_L^* = excited signlet state pigment in live array

TA = ground state of open reaction center

T*A = excited state of reaction center

k_T = rate constant for energy transfer from excited antenna pigment in live array to open reaction center

k_{hl} = rate constant for de-excitation of pigments in live array by thermal losses and spillover to PS I

σ_L = effective absorption cross section (size) of PS II live pigment array

k_{el} = rate constant for energy transfer from live pigment array to fluorescent array

k_f = rate constant for de-excitation of fluorescent array via radiative decay

k_h = rate constant for thermal relaxation of fluorescent array

Chl_F = ground state pigment in fluorescent array

Chl_F^* = excited state pigment in fluorescent array

I_m = intensity of modulated measuring beam

F_L = level of live fluorescence

We can therefore represent the level of live fluorescence, F_L, by the following equation:

$$F_L = [k_f/(k_h + k_f)][(\sigma_L I_m k_{el})/(k_T(TA) + k_{el} + k_{hl})] \quad \text{(Eq. II.C)}$$

As in the case of the two site model, the intensity dependence data of Figure II.5 give us valuable information about the relative values of several of the rate constants in the equation above. The direct proportionality between F_L and actinic intensity requires that $(TA) \alpha 1/I_{act}$ and k_T (TA) >> k_{hl} and k_{el}. The large Mg^{2+} stimulated increase in (total) fluorescence in the presence of DCMU and dithionite, when photochemistry is blocked (TA) $\approx$ 0, indicates that the Mg^{2+} stimulated fluorescence increase does not arise from a decrease in the term k_T (TA) in equation II.C. Making the usual assumption that k_f is constant, we have three possible origins for the Mg^{2+} induced increase in live fluorescence. First, a Mg^{2+} induced increase in σ_L, corresponding to an increase in the size (effective absorption cross section) of the PS II live antenna array, could account for the Mg^{2+} stimulated increase in live fluorescence. Secondly, a Mg^{2+} induced increase in k_{el} would also result in an increase in the leved of live fluorescence. A third possible origin for the Mg^{2+} stimulated live fluorescence increase is a Mg^{2+} induced decrease in k_h. On the basis of our fluorescence results alone, it is not possible to determine which of these three possible explanations for live fluorescence increases of a single site model is correct.

Dead Fluorescence: We can represent the processes involved in determining the level of dead fluorescence by the following equations:

		Rate
Excitation	$Chl_D + h\nu \rightarrow Chl_D^*$	$\sigma_D I_m$
Thermal decay	$Chl_D^* \xrightarrow[k_{hd}]{} Chl_D$ + "heat"	$k_{hd}(Chl_D^*)$
Energy transfer to fluorescent array	$Chl_D^* + Chl_F \xrightarrow[k_{ed}]{} Chl_D + Chl_F^*$	$k_{ed}(Chl_D^*)(Chl_F)$
Fluorescence	$Chl_F^* \xrightarrow[k_f]{} Chl_F + h\nu'$	$k_f(Chl_F^*)$
Thermal relaxation of fluorescent array	$Chl_F^* \xrightarrow[k_h]{} Chl_F$ + "heat"	$k_h(Chl_F^*)$

where Chl_D = ground state pigment in dead array

Chl_D^* = excited singlet state pigment in dead array

Chl_F = ground state pigment in fluorescent array

Chl_F^* = excited singlet state pigment in fluorescent array

k_{hd} = rate constant for de-excitation of pigments in dead array by thermal decay

σ_D = effective absorption cross section (size) of PS II dead array

k_{ed} = rate constant for energy transfer from dead array to fluorescent array

k_f = rate constant for de-excitation of fluorescent array via radiative decay

k_h = rate constant for thermal relaxation of fluorescent array

I_m = intensity of modulated measuring beam

F_D = level of dead fluorescence

We can, therefore, represent dead fluorescence by the following equation:

$$F_D = [k_f/(k_h + k_f)][(\sigma_D I_m k_{ed})/(k_{ed} + k_{hd})] \qquad \text{(Eq. II.D)}$$

Assuming k_f to be constant, a Mg^{2+} induced increase in F_D could arise from an increase in σ_D, k_{ed}, or a decrease in k_h or k_{hd}. The data available at present do not allow us to determine which of these four alternative explanations for Mg^{2+} stimulated increases in dead fluorescence is correct. It should be noted that a decrease in k_h would increase the level of live and dead fluorescence. However, a decrease in k_h alone cannot explain both types of fluorescence increases because of the different concentration dependence observed for the two components of fluorescence.

We have interpreted our experimental results in terms of both a two site model and a single site model because it is not yet clear which of these models is correct or whether other alternative formulations should be considered. It may be somewhat easier to reconcile the observation that live and dead fluorescence show the same wavelengths of emission with a single site model. However, this observation can also be explained by a two site model by proposing that the two sites of emission fortuitously emit radiation of the same wavelength.

Both types of models allow Mg^{2+} induced increases in live fluorescence to be explained in terms of an increase in σ_L and Mg^{2+} increases in dead fluorescence in terms of an increase in σ_D or a decrease in k_{hd}. The major difference in the explanation of fluorescence increases between these two models is that the single site model allows for additional Mg^{2+} action at the points of energy transfer from the absorbing pigment array to the fluorescent pigment array and at the level of thermal decay of the fluorescent pigment array. The quantum yields

of PS II photochemistry (0.5 to 1.0) reported from several laboratories[20,21] indicate that the live pigment array must make up the dominant fraction of PS II chlorophyll in either model.

The only significant experimental evidence which argues against our proposal that Mg^{2+} increases the absorption cross section of PS II chlorophyll is found in the work of Briantais et al.[16] They pre-illuminated samples and then measured both the (total) fluorescence intensity (F) and the lifetime (τ) using a phase fluorometer. They then plotted τ versus F for samples with and without Mg^{2+}. We have reproduced this plot in Figure II.10. Briantais et al.[15] maintain that the same co-linear relationship between τ and F is obtained either in the presence or absence of Mg^{2+}. They concluded that Mg^{2+} induces a real increase in PS II fluorescence yield instead of an increase in the amount of excitation reaching PS II reaction centers. However, our analysis of the data indicates that the following factors must be taken into consideration in evaluating these results.

1. Implicit in the use of a phase fluorometric technique is the assumption that the decay of fluorescence follows a single exponential decay. As has been previously discussed by Müller et al.[22] and Pearlstein,[23] one would have to employ a chopping frequency of about 10^9 Hz to determine the validity of this assumption. However, Briantais et al.[16] used a chopping frequency of 7.25 MHz and were thus unable to determine if their decay was truly exponential.

The report of Seibert and Alfano[24] of two peaks in the sub-nanosecond decay curve of chloroplast fluorescence indicates that chloroplast fluorescence cannot be accurately represented by a single exponential decay.

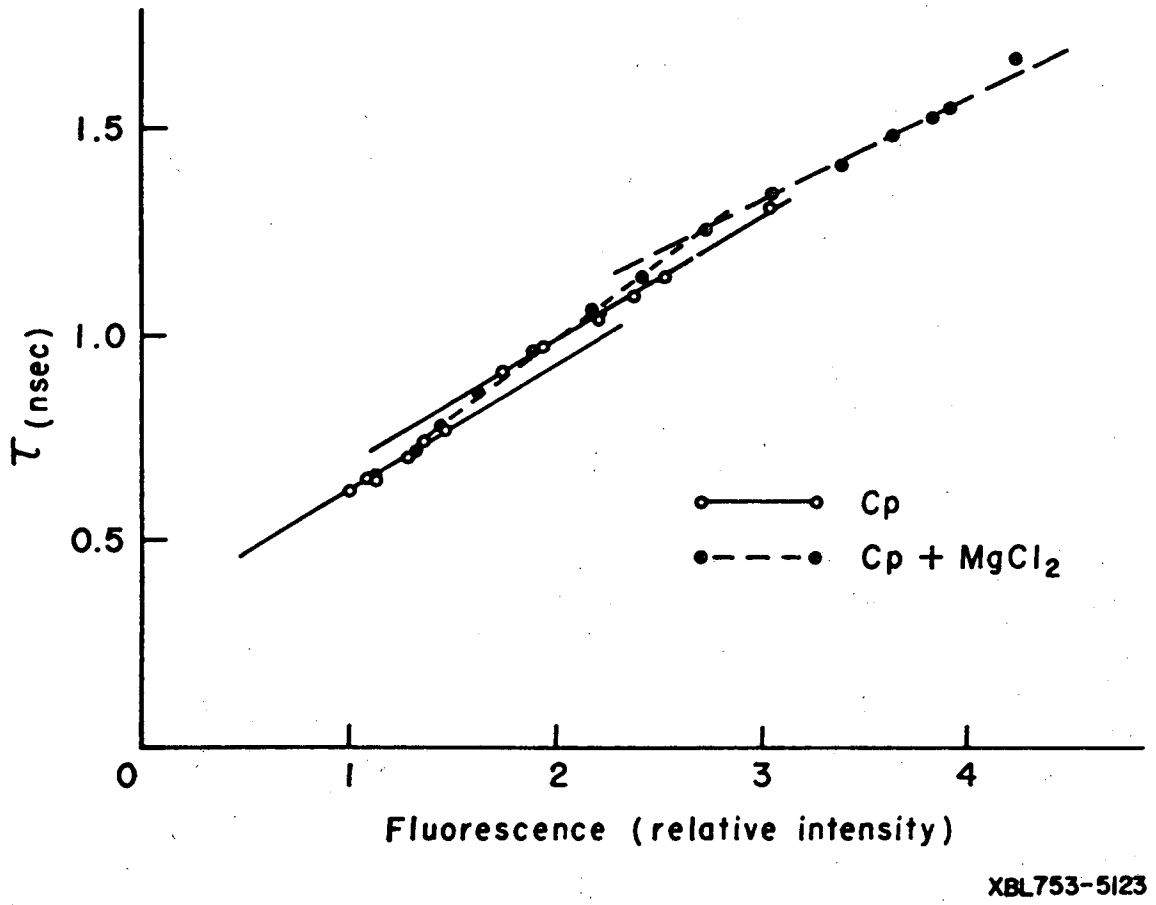

Figure II.10. Effect of $MgCl_2$ on the relationship between chloroplasts (Cp) fluorescence lifetime (τ) and fluorescence intensity. Figure is adapted from reference 16. As described in our text, the figure from reference 16 has been modified by the addition of four straight lines through the data points. For experimental conditions see reference 16.

For a single exponential decay the τ versus F plot should extrapolate to the origin; however, in Figure II.10 we see that F extrapolates to a substantial negative intensity at τ equal to zero.

2. Although other figures in reference 23 show that Mg^{2+} increases the steady-state maximum fluorescence level by a factor of 2.5, in the lifetime study (Fig. II.10) the maximum fluorescence intensity ($-Mg^{2+}$) is only increased by a factor of 1.5 when Mg^{2+} is added to the sample. While it is possible that the differing amount of Mg^{2+} stimulation of fluorescence in these two cases reflects variation in experimental procedures, it is also possible that this difference in Mg^{2+} effect indicates that a significant fraction of the live fluorescence (induced by a pre-illuminating flash) decayed before the lifetime measurement was made.

3. We also question the assertion that the τ versus F plot shows the same co-linear relationship both in the presence and absence of Mg^{2+}. Although we do not have sufficient information about error limits to fit these curves precisely to particular functional forms, we estimate that the data for chloroplasts without Mg^{2+} can best be fit by two non-intersecting lines of the same slope (parallel) rather than by a single line. For chloroplasts with Mg^{2+} the data appear to be fit best by two lines of different slope (see Fig. II.10).

In view of the factors discussed above, we feel that a truly meaningful τ versus F plot requires the direct measurement of fluorescence lifetimes rather than the use of a phase fluorometric technique.

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Chapter III

ABSORPTION SPECTRA AND RELATIVE QUANTUM YIELD STUDIES

Another approach to the problem of determining what role, if any, Mg^{2+} plays in the control of spillover between PS II and PS I involves the measurement of the effect of Mg^{2+} on the quantum yields of PS I and PS II reactions. If the proposal that Mg^{2+} controls spillover between PS II and PS I is correct,[1] we would expect to observe Mg^{2+} induced increases in PS II activity which are accompanied by related Mg^{2+} induced decreases in PS I activity. If Mg^{2+} increases PS II activity through changes which are independent of spillover to PS I then there is no necessary correlation between Mg^{2+} induced changes in PS II activity and PS I activity. Experimentally, we can look at the effect of Mg^{2+} on the rate measured under light limiting conditions (relative quantum yield) of a reaction which involves only PS II, such as $H_2O \rightarrow$ DCIP, and compare it to the Mg^{2+} effect observed for light limited PS I reactions such as DCMU/Asc/TMPD $\rightarrow$ Fd/$NADP^+$ or methyl viologen. Additionally, we can look at how Mg^{2+} affects the light limited rate of a process such as $H_2O \rightarrow$ Fd/$NADP^+$ which involves both PS II and PS I. In the following section we will discuss how Mg^{2+} affects the light limited rates of the reactions listed above. Since interpretation of these results can also be influenced by salt induced changes in absorption spectra, we shall also briefly discuss the effect of Mg^{2+} on chloroplast absorption spectra.

Materials and Methods: Chloroplasts were prepared as described in the fluorescence studies section, with the following two changes. First, approximately tenfold greater chloroplast concentrations (optical density 436 nm = 3-4) were used in these studies. Second, the final chloroplast suspension was filtered through two layers of Miracloth before it was used for optical measurements. Absorption spectra were measured using an AMINCO-Chance DW-2 spectrophotometer operated in the split beam mode. The photomultiplier was positioned 1 cm from the sample and a square of opal glass was inserted between the photomultiplier and the sample to give uniform scattering. DCIP reduction was measured using the DW-2 spectrophotometer in the split beam mode. Actinic illumination from the side was provided by the use of a Tiyoda Microscope Illuminator, Corning 1-69 heat filter, and Optical Industries 480 nm interference filter (bandwidth 10 nm). The rate of DCIP reduction was measured as $\Delta A_{580\ nm}$/time. Measurement bandwidth was 2 nm. The photomultiplier (Hammamatsu R 562 S-20 response) was protected from stray actinic illumination through the use of an Optical Industries 580 nm interference filter which had a bandwidth of 10 nm. $NADP^+$ reduction was measured using either a Cary 14 spectrophotometer equipped with a model 1462 Scattered Transmission Accessory as described by Sun and Sauer[2] or by use of the DW-2 spectrophotometer in the split beam mode. Side actinic illumination was provided in the same manner used for DCIP reduction. The photomultiplier was protected from stray actinic illumination by use of a Kodak Wratten 18A filter. Measurement bandwidth using the DW-2 was 6 nm. The rate of $NADP^+$ reduction was recorded as

$\Delta A_{340\ nm}$/time. Oxygen uptake was measured using the Clark type apparatus previously described by Blankenship and Sauer.[3] An Optical Industries 480 nm interference filter with 10 nm bandwidth plus appropriate heat and water filters was used to isolate the desired wavelength of actinic illumination. In all intensity studies, Balzers calibrated neutral density filters were used in the determination of relative actinic intensities. Unless otherwise specified, all chloroplast samples were dark adapted for at least 30 minutes prior to use in electron transport measurements.

All reagents were obtained from the sources described in the fluorescence section, with the following additions: TMPD was obtained from Eastman Kodak, sodium ascorbate was purchased from Calbiochem, and methyl viologen was obtained from Sigma.

Results - Absorption Spectra: Figure III.1 shows that addition of 5 mM $MgCl_2$ to a chloroplast suspension causes significant decreases in both the Soret and red regions of the absorption spectrum. These absorbance decreases in regions of high optical density most likely result from optical flattening owing to changes in the arrangement of chloroplast membranes. Electron microscope studies[4,5] and light scattering[6] measurements have shown that addition of Mg^{2+} to chloroplast suspensions causes major structural alterations in chloroplast membrane organization. The Mg^{2+} induced absorbance changes seen in Figure III.1 conflict with the data of Murata[1] and Briantais,[7] both of whom reported that addition of $MgCl_2$ did not cause significant changes in the light absorption of the chloroplast. Our observation is supported by the report of Gross et al.[8] that addition of Mg^{2+} to

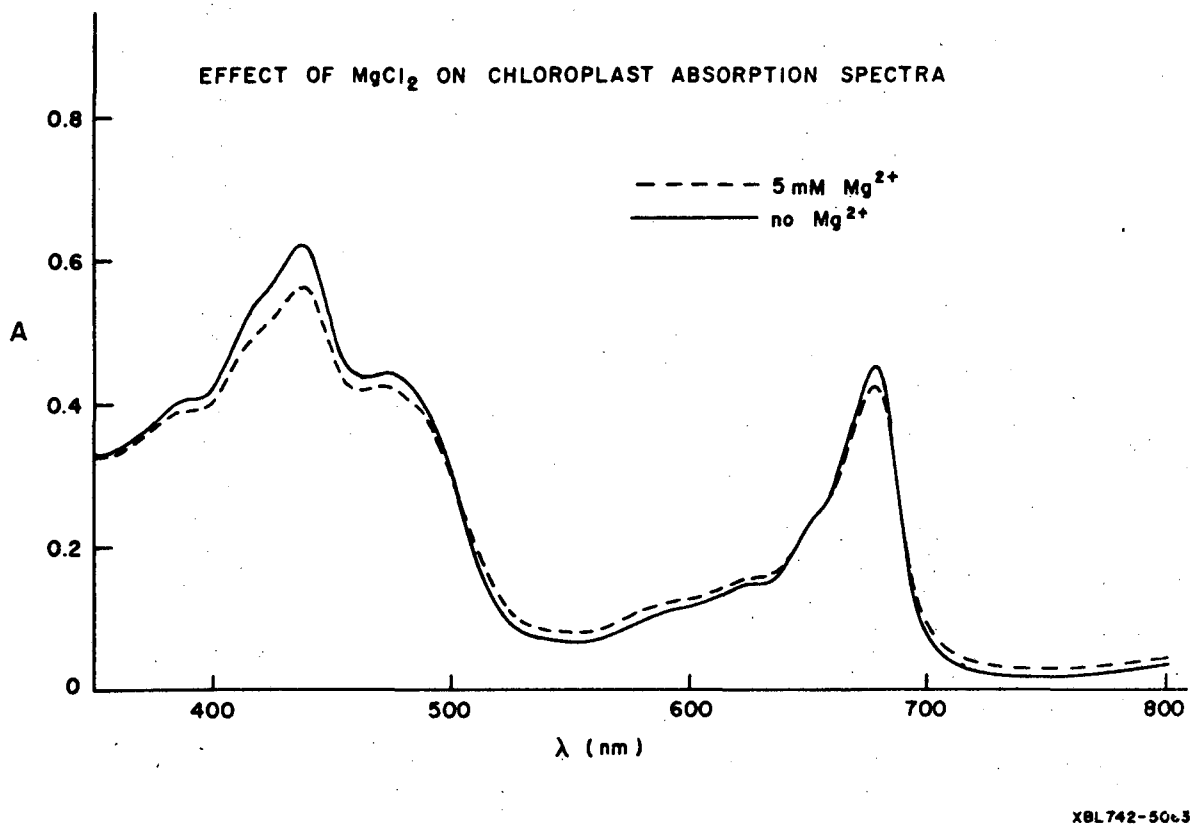

Figure III.1. Effect of $MgCl_2$ on chloroplast absorption spectra. Spectral bandpass, 3.0 nm. Other details as described in text.

chloroplasts causes absorbance decreases in the Soret and red regions of the spectrum.

EFFECT OF Mg^{2+} ON ELECTRON TRANSPORT RATES MEASURED UNDER LIGHT LIMITING CONDITIONS (RELATIVE QUANTUM YIELDS)

PS II Studies: $H_2O \rightarrow$ DCIP. Sun and Sauer[2] reported the (zero intensity) absolute quantum yield of this reaction to be 1.0. This value indicates that this reaction involves only PS II ("pure" PS II reaction). Figure III.2 shows the effect of Mg^{2+} and actinic intensity on the rate of this reaction. For the sample without Mg^{2+} the rate of DCIP reduction (ΔA_{580}/sec) was 0.0002 at a relative intensity (I_{rel}) of 50. At I_{rel} = 100 the rate of DCIP reduction of this sample was 0.0004. The sample containing 7.5 mM Mg^{2+} had a rate of DCIP reduction (ΔA_{580}/sec) of 0.0004 at I_{rel} = 50. The rate of DCIP reduction of this Mg^{2+} containing sample was 0.0008 at I_{rel} = 100. For samples with and without Mg^{2+}, we observe that the rate of DCIP reduction doubles when the actinic intensity doubles; hence we know we are working in a photon limited region of the saturation curve. In this particular experiment we also see that Mg^{2+} doubles the light limited rate of DCIP reduction. As is true with many other reported[1,9,10] Mg^{2+} effects, there is some variation in the size of the Mg^{2+} stimulation of rate from one sample preparation to another. In our experiments, the Mg^{2+} induced increase in the rate of DCIP reduction ranged from about 35 to 120%. Fresh chloroplasts (less than 2 hours old) prepared from 21 to 27 day old pea plants showed the greatest Mg^{2+} stimulation of DCIP reduction. The average Mg^{2+} induced increase in the rate of

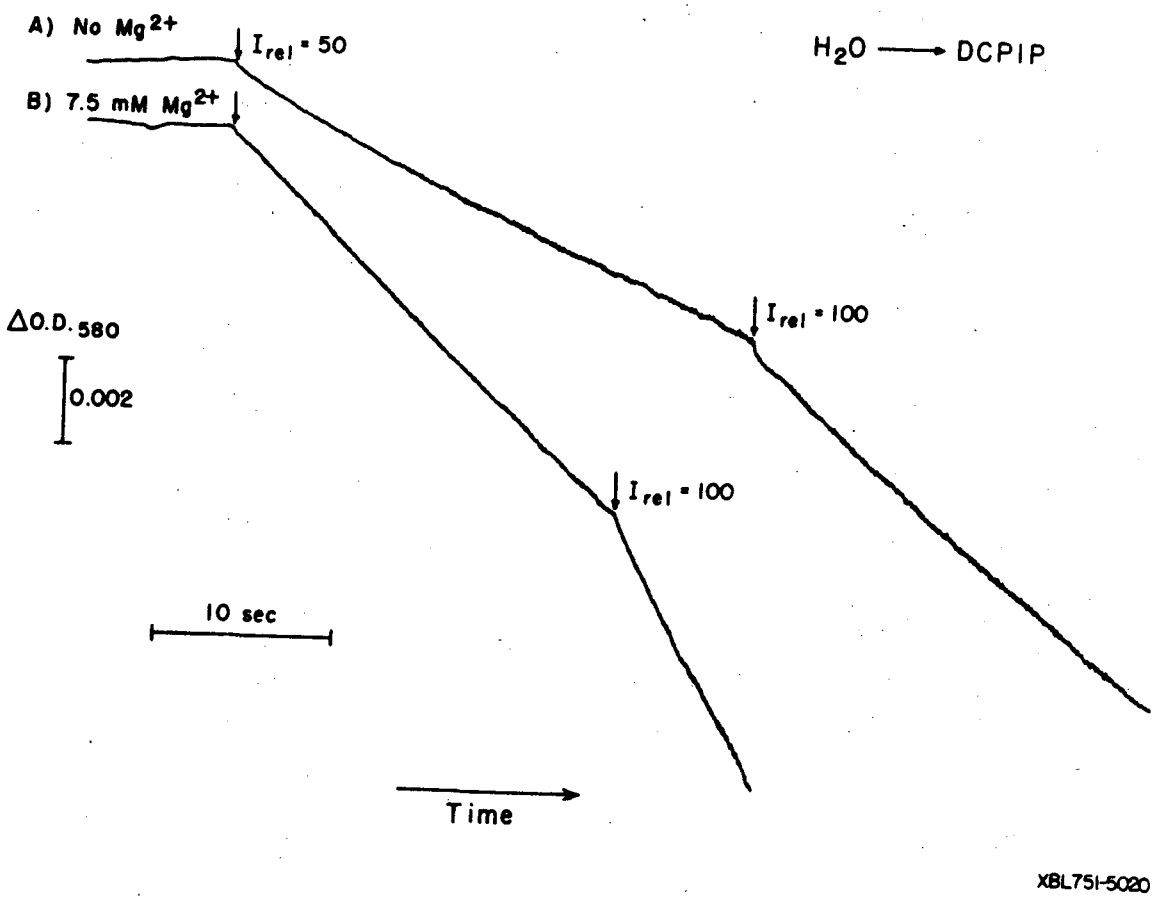

Figure III.2 Effect of Mg^{2+} and actinic intensity on the rate of DCIP reduction. Actinic wavelength, 480 nm; actinic intensity (I_{rel}=100), 60 μw/cm^2; DCIP concentration, 0.02 mM. Other experimental conditions as discussed in text. ↓ indicates start of actinic illumination at the I_{rel} value shown on the figure.

DCIP reduction was computed to be 76% ± 7% for a total of 18 pairs of dark adapted samples. For the $H_2O \rightarrow$ DCIP data shown in Figure III.2 we have computed a quantum yield (equivalents/einstein) of 0.3 for the sample without Mg^{2+} and 0.6 for the sample with Mg^{2+}. Quantum yield values ranging from 0.26[1] to 1.0[2] have been reported in previous studies of the $H_2O \rightarrow$ DCIP reaction in spinach chloroplasts. It is interesting to note that the Mg^{2+} induced increase in the light limited rate of this PS II reaction is of about the same magnitude as the Mg^{2+} induced increase in the slope of the F_V vs. I_{act} plot in the linear intensity region (Figure II.5). At higher intensities, where the rate is not strictly photon limited, Sun and Sauer[2] reported Mg^{2+} increased the rate of indophenol reduction by about 60%. Although both our studies and those of Murata[1] used the same wavelength of actinic illumination (480 nm), we observe a degree of Mg^{2+} stimulation of light limited indophenol reduction that is significantly (7-fold) greater than that reported by Murata.[1] Using 640 nm actinic illumination, Briantais et al.[7] reported that Mg^{2+} causes about a 40% increase in the light limited rate of the indophenol Hill reaction. It should be noted that the experiments of Sun and Sauer,[6] Murata,[1] and Briantais et al.[7] were carried out on spinach chloroplasts, whereas our experiments were performed on pea chloroplasts.

Working with lettuce chloroplasts, Avron and Ben-Hayyim measured the absolute zero intensity quantum yield of the $H_2O \rightarrow$ ferricyanide reaction and reported it to be unity. Hence they concluded that this reaction involved PS II solely. These authors then measured the effect of Mg^{2+} on this reaction under light limiting (640 nm) conditions

and reported that addition of Mg^{2+} increased the light limited Hill rate by 75%.[12] This result is in reasonable agreement with our results with DCIP as the terminal electron acceptor. More recently, however, Marsho and Kok, using spinach chloroplasts, and Harnischfeger and Shavit,[13] using lettuce chloroplasts, have reported that Mg^{2+} does not significantly affect the rate of the $H_2O \rightarrow$ ferricyanide reaction. The reason for the discrepancy between these results is not yet known.

PS I Reactions:

A. DCMU/Asc/TMPD $\rightarrow$ Fd/$NADP^+$: Since electron transport from reduced TMPD to $NADP^+$ proceeds efficiently in the presence of the oxygen evolution inhibitor DCMU, we can safely conclude that this reaction involves PS I alone. The effects of actinic intensity and Mg^{2+} addition on the rate of this reaction are seen in Figure III.3. In contrast to the DCIP Hill reaction, the Asc/TMPD $\rightarrow$ $NADP^+$ reaction requires a relatively long induction period in this intensity region before it reaches its steady-state level. This induction period is particularly noticeable for samples which lack Mg^{2+}. For the sample without Mg^{2+} we observe a steady-state rate of $NADP^+$ reduction (ΔA_{340}/sec) of 1.2×10^{-4} at $I_{rel} = 50$. At $I_{rel} = 100$ this sample had a rate of $NADP^+$ reduction of 2.4×10^{-4}. The sample with 7.5 mM Mg^{2+} had a rate of $NADP^+$ reduction of 1.7×10^{-4} at $I_{rel} = 50$. This Mg^{2+} containing sample had a rate of $NADP^+$ reduction of 3.3×10^{-4} at $I_{rel} = 100$. For samples with and without Mg^{2+}, we therefore observe that the steady-state rate of $NADP^+$ reduction is light limited; hence changes in rate should reflect changes in the primary quantum yield of photochemistry. For the particular experiment shown in

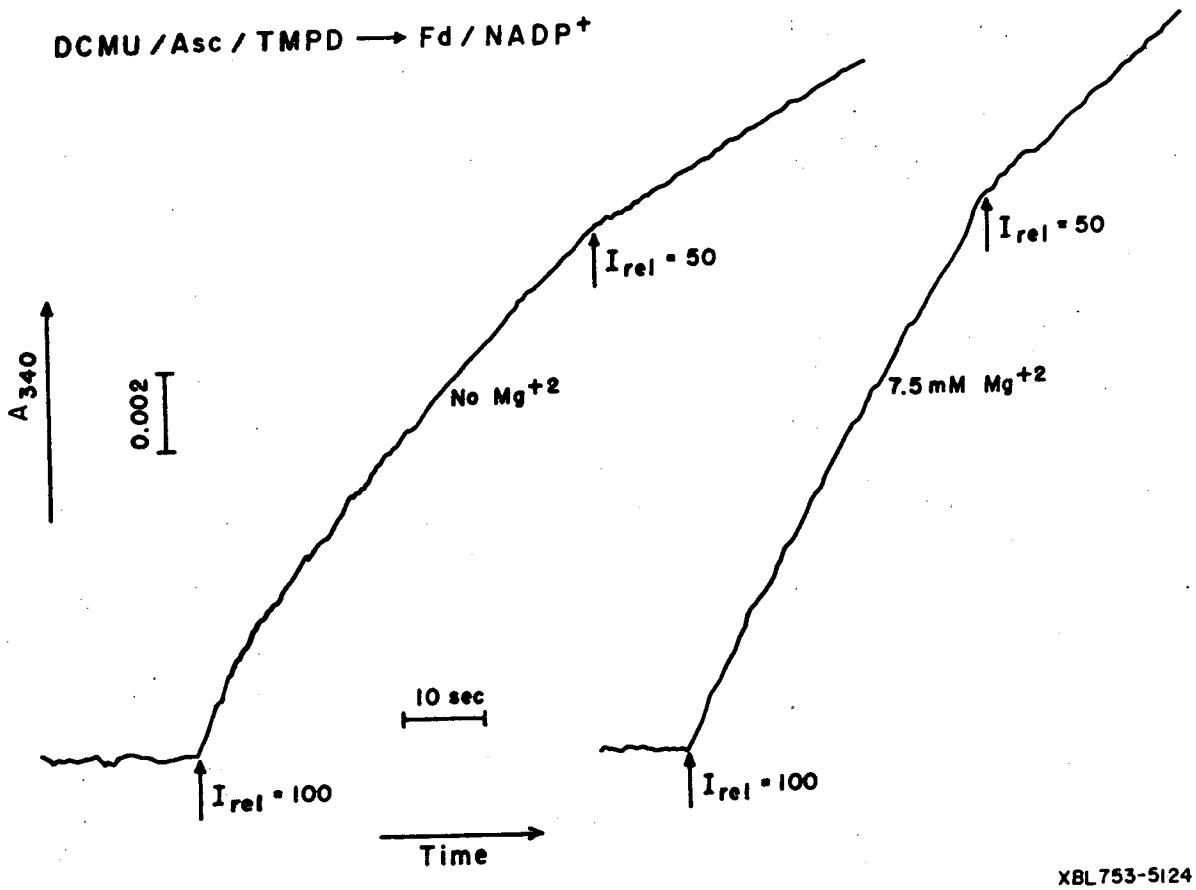

Figure III.3. Effect of Mg^{2+} and actinic intensity on the rate of the DCMU/Asc/TMPD → Fd/$NADP^+$ reaction. DCMU concentration, 0.01 mM; Asc concentration, 40 μg/ml; $NADP^+$ concentration, 0.6 mM. Actinic wavelength and intensity conditions as in Figure III.2. ↑ denotes start of actinic illumination at I_{rel} value indicated on the figure.

Figure III.3, Mg^{2+} caused about a 60% increase in the light limited rate of $NADP^+$ reduction. Mg^{2+} stimulation of rate was obtained with both dark adapted and preilluminated chloroplast samples. As with other Mg^{2+} effects, there was considerable variation in the degree of Mg^{2+} stimulation of $NADP^+$ reduction among different chloroplast samples. Some samples showed less than 10% stimulation of rate upon addition of Mg^{2+}, while other samples showed Mg^{2+} induced increases of 120%. The average increase for a series of 18 pairs of measurements was 52%. Marsho and Kok[10] recently reported that Mg^{2+} stimulated the light limited rate of the Asc/DCIP (or DAD) → $NADP^+$ reaction. They also reported considerable variation (40-100%) in the size of Mg^{2+} induced increases. Significantly, neither we nor Marsho and Kok,[10] when also using ferredoxin from Sigma, ever observed a Mg^{2+} induced <u>decrease</u> for this type of pyridine nucleotide reduction reaction. Harnischfeger and Shavit[13] also report stimulation of the Asc/DCIP → Fd/$NADP^+$ reaction; however, they do not indicate where they are operating on the intensity saturation curve. (Our studies of the effect of Mg^{2+} on the rate of $NADP^+$ reduction under light saturating conditions will be discussed in a later chapter.)

In contrast to these reports of Mg^{2+} stimulation of NADP reduction, Avron and Ben-Hayyim[11] had earlier reported about a 20% decrease in the light limited rate of the Asc/DCIP → Fd/$NADP^+$ reaction when Mg^{2+} was present. Murata[1] also reported that Mg^{2+} decreased the relative light limited quantum yield of the Asc/DCIP → Fd/$NADP^+$ reaction. Marsho and Kok[10] have suggested that the discrepancies among these reports may result from different sources of ferredoxin and different concentrations of monovalent cations in the medium.

B. DCMU/Asc/TMPD → methyl viologen: In aerobic samples it is not possible to measure directly the rate of methyl viologen reduction because reduced methyl viologen is rapidly oxidized by air. Instead of measuring the rate of methyl viologen photoreduction, one therefore measures the rate at which O_2 is consumed in the oxidation of reduced methyl viologen. Limitations in cell geometry and electrode stability did not allow us to determine if our measurements of O_2 uptake (Table III.1) were actually being done under light limited conditions. Therefore, in Table III.1 we cannot rigorously relate the 20-30% decrease in the rate of O_2 uptake caused by Mg^{2+} to a corresponding change in the excitation density at the PS I reaction center. However, the low actinic intensities and high optical densities used in this experiment make it likely that we are near, if not in, the photon limited region of the saturation curve. Furthermore, our results agree well with the light limited results of Marsho and Kok,[10] who reported Mg^{2+} decreases the relative quantum yield of the Asc/DCIP → methyl viologen reaction by 20%. Briantais et al.[7] reported that under light limiting conditions Mg^{2+} decreased the rate of the Asc/DCIP → methyl viologen reaction by 15-33%. Avron and Ben Hayyim[11] have reported that Mg^{2+} causes a 20% decrease in the light limited rate of the Asc/DCIP → diquat reaction. Harnischfeger and Shavit,[13] working in an unspecified intensity region, report essentially no effect of Mg^{2+} on the Asc/DCIP → methyl viologen rate when KCl is not present in the reaction mixture. In the presence of 16 to 100 mM KCl, they report Mg^{2+} decreases the rate of the Asc/DCIP → methyl viologen reaction by about 30%. In their study of Mg^{2+} effects on Euglena

Table III.1

Effect of Mg^{2+} on the Rate of O_2 Uptake for the DCMU/Asc/TMPD Methyl Viologen Reaction

Experimental conditions: excitation wavelength, 480 nm; Asc, 2.5 mM; TMPD, 0.2 mM; $MgCl_2$, 7.5 mM; methyl viologen, 1 mM; KCN, 0.1 mM; DCMU, 0.01 mM; other experimental conditions for chloroplast preparation and use of electrode as described in text

	Rel. Rate (no Mg^{2+})	Rel. Rate ($+Mg^{2+}$)	Rate $\frac{-Mg^{2+}}{+Mg^{2+}}$
Expt. #1	32 ± 1	26 ± 1	1.23
Expt. #2	33 ± 1	27 ± 1	1.26
Expt. #3	38 ± 1	30 ± 1	1.27

chloroplasts, Jennings and Forti[14] showed that Mg^{2+} greatly stimulated Euglena chloroplast fluorescence but did not affect the rate of the Asc/DCIP → methyl viologen reaction.

PS I + PS II:

$H_2O \rightarrow NADP^+$: The existence of Emerson Enhancement[15] for this reaction clearly indicates that it requires the sequential participation of PS II and PS I. This requirement for participation of both photoreactions is consistent with the quantum yield of 0.5 reported for this reaction by Sun and Sauer[2] and Avron and Ben-Hayyim.[11] As shown in Figure III.4, Mg^{2+} markedly stimulates the light limited rate of the $H_2O \rightarrow NADP^+$ reaction. The average Mg^{2+} induced increase in the light limited rate of $NADP^+$ reduction was 130%. This Mg^{2+} induced increase in the light limited rate of pyridine nucleotide reduction may be compared with the data of Marsho and Kok,[10] who reported Mg^{2+} stimulated this rate by 40-100%. Rurainski, Randles and Hoch,[16] using uncoupled chloroplasts, also reported that Mg^{2+} increases the light limited rate of the $H_2O \rightarrow NADP^+$ reaction by about 100%. These experiments of Rurainski et al.[16] indicate that the Mg^{2+} stimulation of light limited $NADP^+$ reduction is distinct from the Mg^{2+} requirement for phosphorylation in coupled chloroplasts. Harnischfeger and Shavit,[13] working in an unspecified intensity region, recently also reported Mg^{2+} stimulation of the $H_2O \rightarrow NADP^+$ reaction. Their data indicates that maximal Mg^{2+} stimulation of rate (sixfold) occurs in the absence of KCl. In the presence of 16 mM KCl, these authors report that Mg^{2+} only stimulates the rate of $NADP^+$ reduction in both coupled and uncoupled chloroplasts.

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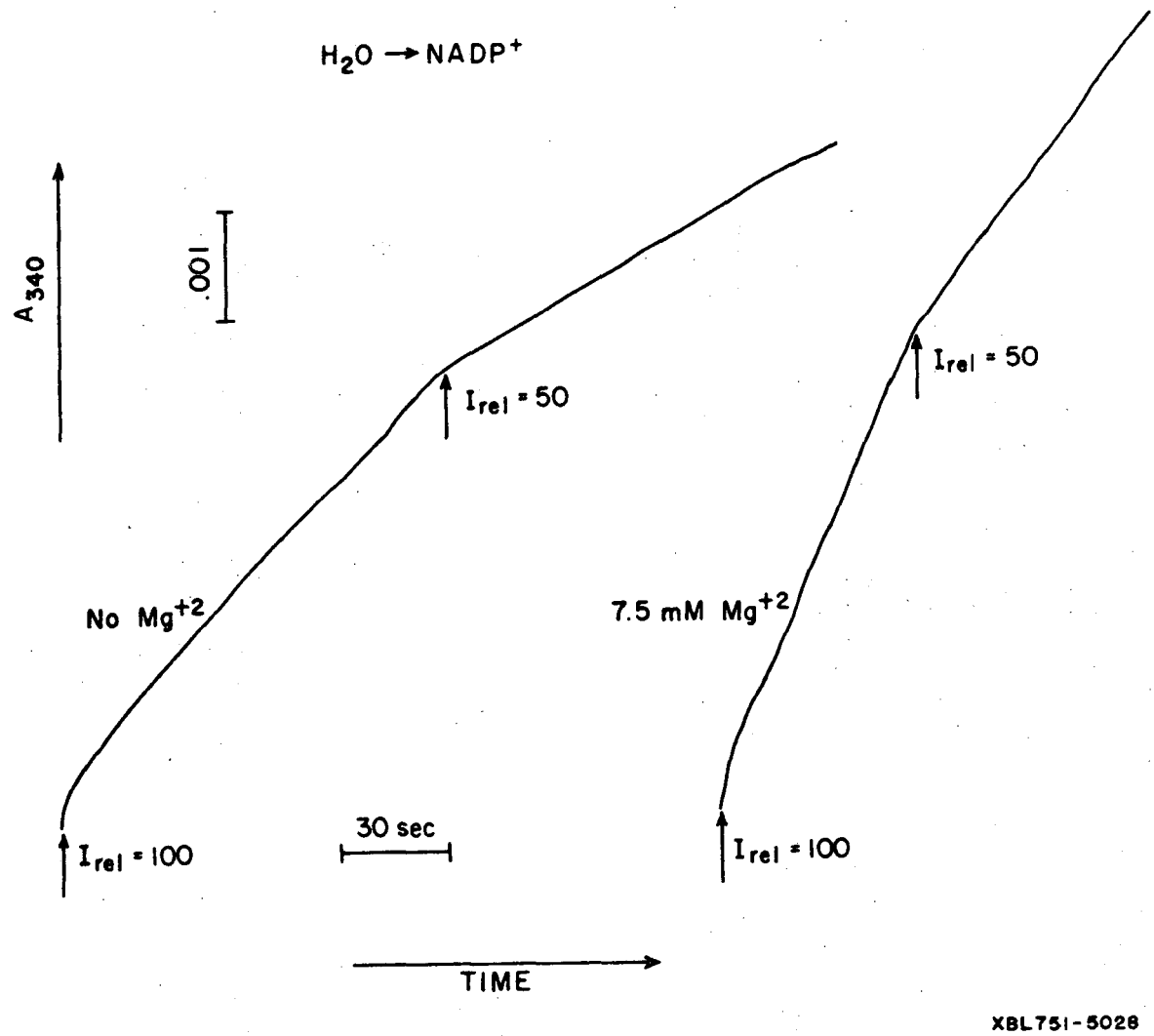

Figure III.4. Effect of Mg^{2+} and actinic intensity on the rate of the $H_2O \rightarrow NADP^+$ reaction. Illumination conditions as in Figure III.3; Fd concentration, 40 μg/ml; $NADP^+$ concentration, 0.6 mM. ↑ indicates start of actinic illumination at I_{rel} value shown on figure.

Marsho and Kok[10] showed that they could observe Emerson Enhancement in the $H_2O \rightarrow$ methyl viologen reaction, as well as for the $H_2O \rightarrow NADP^+$ reaction. This result indicated that electron flow to methyl viologen also required sequential participation of both photoreactions. However, in contrast to the $H_2O \rightarrow NADP^+$ reaction, the relative quantum yield of the $H_2O \rightarrow$ methyl viologen reaction was reported to be unaffected by the presence of Mg^{2+}.[10]

Discussion: Using spinach chloroplasts, Sun and Sauer[6] reported a quantum yield of 1.0 for the $H_2O \rightarrow$ DCIP reaction. This result was interpreted as indicating that DCIP reduction involves electron flow through PS II alone. Assuming that DCIP reduction in our pea chloroplasts also involves PS II alone, we can use the rate of DCIP reduction as a measure of PS II photochemistry. The rate of PS II photochemistry is determined by the following processes:

PS II Photochemistry

Process		Rate
Excitation	$Chl_L + h\nu \rightarrow Chl_L^*$	$\sigma_L I_{act}$
Trapping & Photochemistry	$Chl_L^* + TA \xrightarrow{k_T} Chl_L + T^*A \xrightarrow[chem]{photo-} T^+A^-$	$k_T(Chl_L^*)(TA)$
Non-radiative decay‡	$Chl_L^* \xrightarrow{k_{hl}} Chl_L + \text{"heat"}$	$k_{hl}(Chl_L^*)$
Fluorescence	$Chl_L^* \xrightarrow{k_{fl}} Chl_L + h\nu'$	$k_{fl}(Chl_L^*)$

‡ Non-radiative decay includes possible energy transfem to PS I and to fluorescent pigment arrays. (We also assume that once excitation reaches an open reaction center it has essentially unit probability of being utilized for electron transfer.)

where Chl_L^* = excited PS II antenna pigment which can transfer excitation to the reaction center

Chl_L = ground state of PS II antenna pigment (pigment in live array)

TA = ground state of PS II reaction center

T*A = excited state of PS II reaction center

T^+A^- = photochemically produced products

I_{act} = intensity of actinic illumination

k_T = rate constant for energy transfer from antenna pigment to open reaction center

k_{hl} = rate constant for non-radiative decay of antenna pigments

k_{fl} = rate constant for fluorescent decay of antenna pigments

σ_L = effective absorption cross section (size) of PS II antenna

V_{II} = rate of PS II photochemistry

We can therefore represent the rate of trapping (& photochemistry) as $V_{II} = (k_T(TA)\ \sigma_L\ I_{act})/(k_T(TA) + k_{hl} + k_{fl})$. The doubling of rate of DCIP reduction upon doubling of actinic intensity (direct proportionality) seen in Figure III.2 indicates that the quantity $\frac{k_T(TA)}{k_T(TA) + k_{hl} + k_{fl}}$ must be independent of I_{act}. As discussed in the fluorescence section, changes in I_{act} cause changes in (TA). Therefore we would observe $\frac{k_T(TA)}{k_T(TA) + k_{hl} + k_{fl}}$ independent of I_{act} when $k_T(TA) >> k_{hl}, k_{fl}$ or when $k_T(TA) << k_{hl}, k_{fl}$. The relatively high quantum yields (0.3 → 0.6) which we have obtained for this reaction that we are operating in a situation where $k_T(TA) >> k_{hl}, k_{fl}$. Under these conditions we can write that $\frac{k_T(TA)}{k_T(TA) + k_{hl} + k_{fl}} \approx 1$.

We can therefore approximate V_{II} as σ_L I_{act}. Using this expression, we see that at a fixed actinic intensity changes in rate (V_{II}) correspond to changes in σ_L. In particular, the Mg^{2+} induced doubling of light limited rate (Figure III.2) of DCIP reduction apparently corresponds to a Mg^{2+} induced doubling of σ_L. As discussed in the fluorescence section, increases in σ_L can result from increased spillover from PS I to PS II or from "connection" of previously "disconnected" chlorophyll to PS II. Since our data requires that $k_T(TA) >> k_{hl}$, it is clear that a Mg^{2+} induced blockage of PS II → PS I spillover cannot account for the Mg^{2+} induced doubling of the light limited rate of DCIP reduction seen in Figure III.2. The similarities in the magnitude of the Mg^{2+} induced increase in live fluorescence (Figure II.5) and PS II photochemistry (Figure III.2) strongly suggest to us that both of these effects result from a Mg^{2+} induced in σ_L.

<u>DCMU/Asc/TMPD → $NADP^+$</u>:

The following factors should be considered in the interpreting of the Mg^{2+} stimulated increase in the light-limited rate of this reaction (Figure III.3).

1. A Mg^{2+} induced increase in excitation density at the PS I trap would increase the light-limited rate of PS I photochemistry. This Mg^{2+} stimulated increase in excitation density at the PS I trap could reflect a Mg^{2+} induced increase in the effective absorption cross section (size) of the PS I antenna. A Mg^{2+} induced increase in excitation density at the PS I trap could also reflect a Mg^{2+} stimulated increase in the efficiency of energy transfer from the PS I antenna to the PS I trap.

2. Since ferredoxin is a cofactor of both cyclic and non-cyclic electron flow, a change in the fraction of electrons which are transferred by Fd to $NADP^+$, relative to the fraction of electrons used in cyclic flow, would affect the light limited rate of $NADP^+$ reduction. In particular, a Mg^{2+} induced increase in the non-cyclic/cyclic* ratio would account for a Mg^{2+} induced increase in the light limited rate of $NADP^+$ reduction.

3. Marsho and Kok,[10] and Harnischfeger and Shavit,[13] have previously suggested that Mg^{2+} stimulates the rate of $NADP^+$ reduction by increasing the rate of an electron transfer step on the "dark" electron transport chain which connects Fd to $NADP^+$. While this type of interaction may be significant under moderate intensity conditions, under light-limiting conditions Mg^{2+} control of dark electron transfer steps should not influence the rate of $NADP^+$ reduction.

On the basis of our DCMU/Asc/TMPD → $NADP^+$ results alone we cannot distinguish between the first two explanations for Mg^{2+} stimulation of $NADP^+$ reduction. However, it is difficult to reconcile the Mg^{2+} induced increases in excitation density predicted by explanation 1 with the Mg^{2+} induced decreases in the rate of the DCMU/Asc/TMPD → methyl viologen reaction (Table III.1) because the factors which increase the excitation density should, to a first approximation, be independent of the nature of the terminal electron acceptor. In contrast, it is quite reasonable to assume that in the presence of different electron acceptors, such as Fd/$NADP^+$ and methyl viologen, that there will be a different

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*Non-cyclic/cyclic refers to the ratio of PS I electrons used for non-cyclic flow to that used for cyclic flow.

distribution electron flow between cyclic and non-cyclic pathways. Furthermore, since methyl viologen is chemically quite different from Fd, it would not be surprising to observe different Mg^{2+} interactions with different acceptors.

DCMU/Asc/TMPD → methyl viologen:

As discussed above, the light-limited rate of PS I photochemistry is sensitive to the non-cyclic/cyclic ratio and to the excitation density at the PS I trap. We should therefore consider the following factors in interpreting the data of Table III.1.

1. The Mg^{2+} induced decrease in the rate of the DCMU/Asc/TMPD → methyl viologen reaction may indicate that the non-cyclic/cyclic ratio is decreased by Mg^{2+} when methyl viologen is present in the reaction mixture.

2. The Mg^{2+} induced decrease in the rate of oxygen uptake seen in Table III.1 could also result from a Mg^{2+} induced decrease in excitation density at the PS I trap.

3. An inhibitory effect of Mg^{2+} on the rate of methyl viologen oxidation by oxygen. In recent years, the air oxidation of methyl viologen has been shown to be a rather complex process which involves the formation of a superoxide radical and the break-up of this radical by the enzyme superoxide dismutase. Since there have been no studies carried out as yet on the interaction of Mg^{2+} with the superoxide system, it is not possible at present to evaluate the significance of this type of interaction.

Explanation 2, which predicts lower rates of all PS I photochemistry in the presence of Mg^{2+}, is by itself unable to account for the Mg^{2+} induced increases in PS I photochemistry obtained when Fd/$NADP^+$

replaces methyl viologen as the acceptor system. Of course it is possible that Mg^{2+} affects both the excitation density at the PS I trap and the non-cyclic/cyclic ratio.

<u>$H_2O \rightarrow NADP^+$</u>:

Since this reaction involves participation of both photosystems, it is likely that the Mg^{2+} stimulation of light-limited rate seen in Figure III.4 results from a combination of factors that have previously been discussed for the $H_2O \rightarrow$ DCIP reaction and for the Asc/TMPD $\rightarrow$ Fd/$NADP^+$ reaction.

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Chapter IV

MAGNESIUM ION EFFECTS ON PHOTOSYSTEM I ELECTRON TRANSPORT AS MEASURED BY P700 PHOTO-OXIDATION AND $NADP^+$ REDUCTION

In the previous two chapters we have concentrated primarily on how Mg^{2+} affects energy utilization and electron transport associated with PS II. In this chapter we concentrate on how Mg^{2+} affects processes that control electron transport through PS I. In particular, we have studied how Mg^{2+} affects the degree of photo-oxidation of the PS I trap (P700). In addition, we have also monitored how Mg^{2+} affects the rate of accumulation of reducing equivalents by PS I for different donor systems. Although our experiments suggest several possible sites of Mg^{2+} interaction with PS I, it has become clear that additional experiments will be needed further to localize Mg^{2+} action within PS I.

Materials and Methods: Chloroplasts were prepared as described in the previous chapter except that the samples used in these experimental measurements had an A_{679} of 1.2 → 1.5. Steady state photo-oxidation of P700 was measured using an AMINCO-Chance DW-2 Dual Wavelength Spectrophotometer operated in the dual wavelength mode.[1] Unless otherwise specified, the reference wavelength was 720 nm (bandwidth 2 nm) and the measurement wavelength was 703 nm (bandwidth 2 nm). The time constant of the instrument was 300 msec. Unmodulated actinic illumination was provided by a Tiyoda Microscope Illuminator and an Optical Industries 430 nm interference filter

(bandwidth 10 nm) and a heat filter. Illumination from the actinic source (65 $\mu w/cm^2$) impinged on the cuvette perpendicular to the direction of the modulated measuring and reference beams. In order to eliminate artifacts which could arise from stray 430 nm illumination hitting the photomultiplier and fluorescence artifacts, it was necessary to insert 3 Schott RG 695 filters between the sample cuvette and the photomultiplier. In order to minimize distortion of spectral changes due to scattering and fluorescence artifacts, we positioned the photomultiplier 2 cm from the sample cuvette. A schematic representation of the apparatus used in these P700 measurements is shown in Figure IV.1. $NADP^+$ reduction was measured as described in Chapter III except that the wavelength of actinic illumination was 430 nm instead of 480 nm, and the actinic intensity was 250 $\mu W/cm^2$.

Results: Effect of Mg^{2+} on the Light Induced P700 Absorbance Change:

A. Studies with H_2O as the Electron Donor: To show that the absorbance change we were looking at as $\Delta A_{703-720}$ was indeed due to the oxidation of P700, it was necessary to study the wavelength dependence of this light induced absorbance change. Due to the extremely small absorbance changes ($\Delta A \leq 10^{-3}$) involved it was necessary to obtain the difference spectrum by measuring the absorbance difference at each wavelength under study with a fresh dark-adapted sample. The results of this experiment for the $H_2O \rightarrow$ methyl viologen system are shown in Figure IV.2. We see that the difference spectrum does show the spectral behavior, including the maximum absorbance difference at 703 nm,[1] which is characteristic of P700 oxidation.

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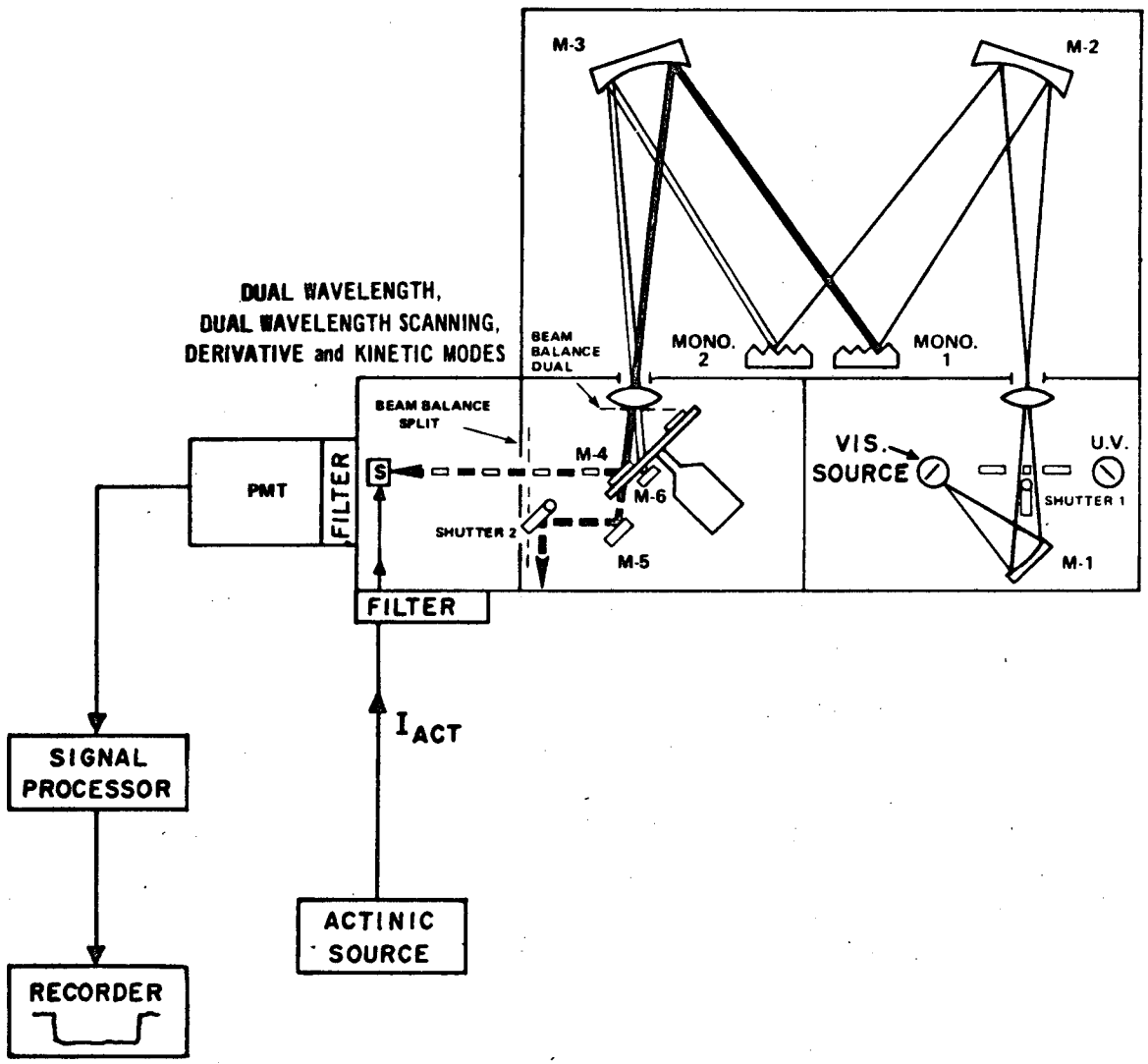

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Figure IV.1. Optical diagram for dual wavelength measurement of P700 photo-gradient. M-1 through M-6 are mirrors. S represents sample cell. PMT represents photomultiplier (HTV R 562). Other details as described in text. (Figure adapted from AMINCO-Chance DW-2 Instrument Manual, American Instrument Co., Silver Spring, Maryland).

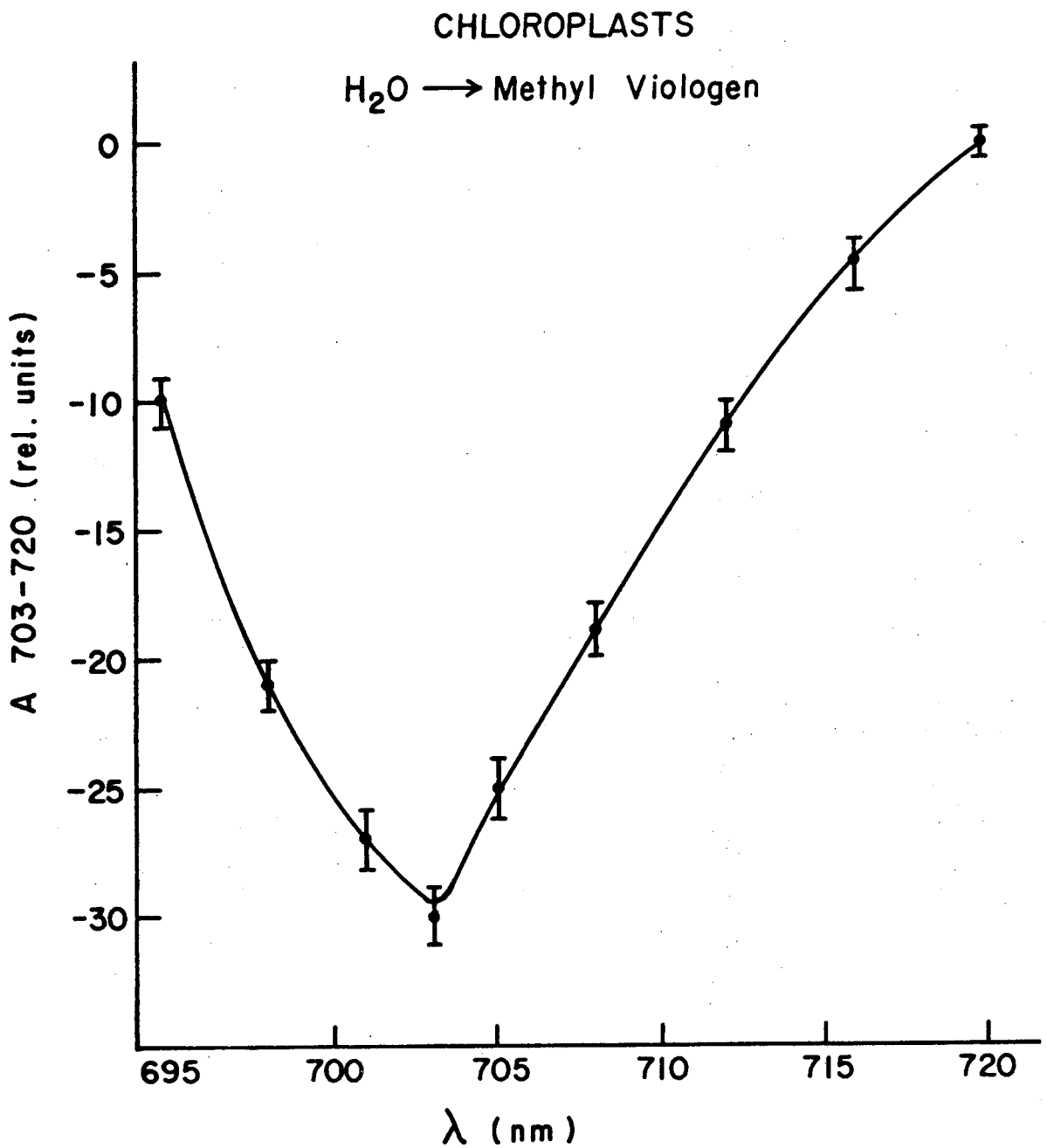

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Figure IV.2. Wavelength dependence of P700 photo-bleaching. Actinic wavelength, 430 nm; actinic intensity, 65 μw/cm^2; methyl viologen concentration, 0.1 mM. At each wavelength shown on the figure, a fresh dark-adapted sample was used to measure the degree of photo-bleaching. Other experimental conditions as discussed in text.

Figure IV.3 shows that for the $H_2O \rightarrow$ methyl viologen reaction the addition of 7.5 mM Mg^{2+} causes a significant (∿35%) decrease in the amount of photo-oxidized P700. Similar effects of 7.5 mM Mg^{2+} are observed when H_2O is the ultimate electron donor and Fd/$NADP^+$ serves as the terminal electron acceptor (Figure IV.4). Additional experiments on the $H_2O \rightarrow$ methyl viologen system (data not shown) indicate that the actinic intensities used are sufficient to saturate the light induced P700 change in the presence and absence of Mg^{2+}. The Mg^{2+} concentration dependence of the Mg^{2+} induced quenching of the P700 photobleaching is shown in Figure IV.5a. We observe that a Mg^{2+} concentration of about 3 mM is sufficient to cause maximum quenching of the P700 photobleaching. Using an amplitude phase shift method rather than a steady state technique to study the $H_2O \rightarrow NADP^+$ system, Rurainski et al.[2] reported that Mg^{2+} decreased the amount of P700 photobleaching by about 80%. They reported that the Mg^{2+} induced quenching of the P700 photobleaching saturated at a Mg^{2+} concentration of 2.5 mM. Recently, Marsho and Kok[3] studied how Mg^{2+} affects the far red illumination induction kinetics of P700 photobleaching in chloroplasts which had previously been illuminated with weak 650 nm light. For the H_2O methyl viologen system, they observed that Mg^{2+} does not alter the magnitude of the steady state P700 photobleaching. However, samples which contain Mg^{2+} require a longer time to reach the steady state P700 level than do the samples which lack Mg^{2+}. They reported that the Mg^{2+} effect on the far red induction kinetics of the P700 photobleaching saturates at a Mg^{2+} concentration of ∿3 mM.

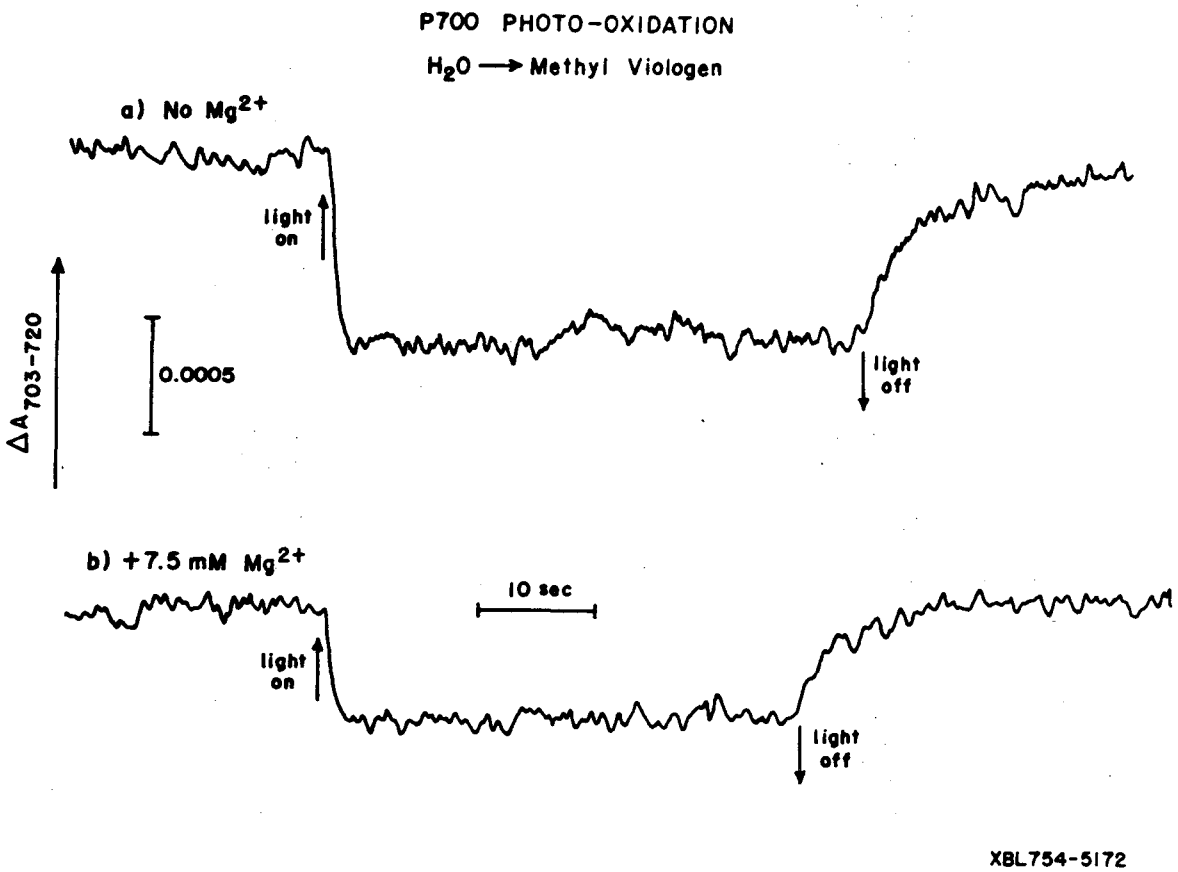

Figure IV.3 Effect of Mg^{2+} on P700 photo-oxidation in the $H_2O \rightarrow$ methyl viologen system. Illumination conditions as in Figure IV.2; methyl viologen concentration, 0.1 mM. Fresh dark-adapted samples were used in both a) and b).

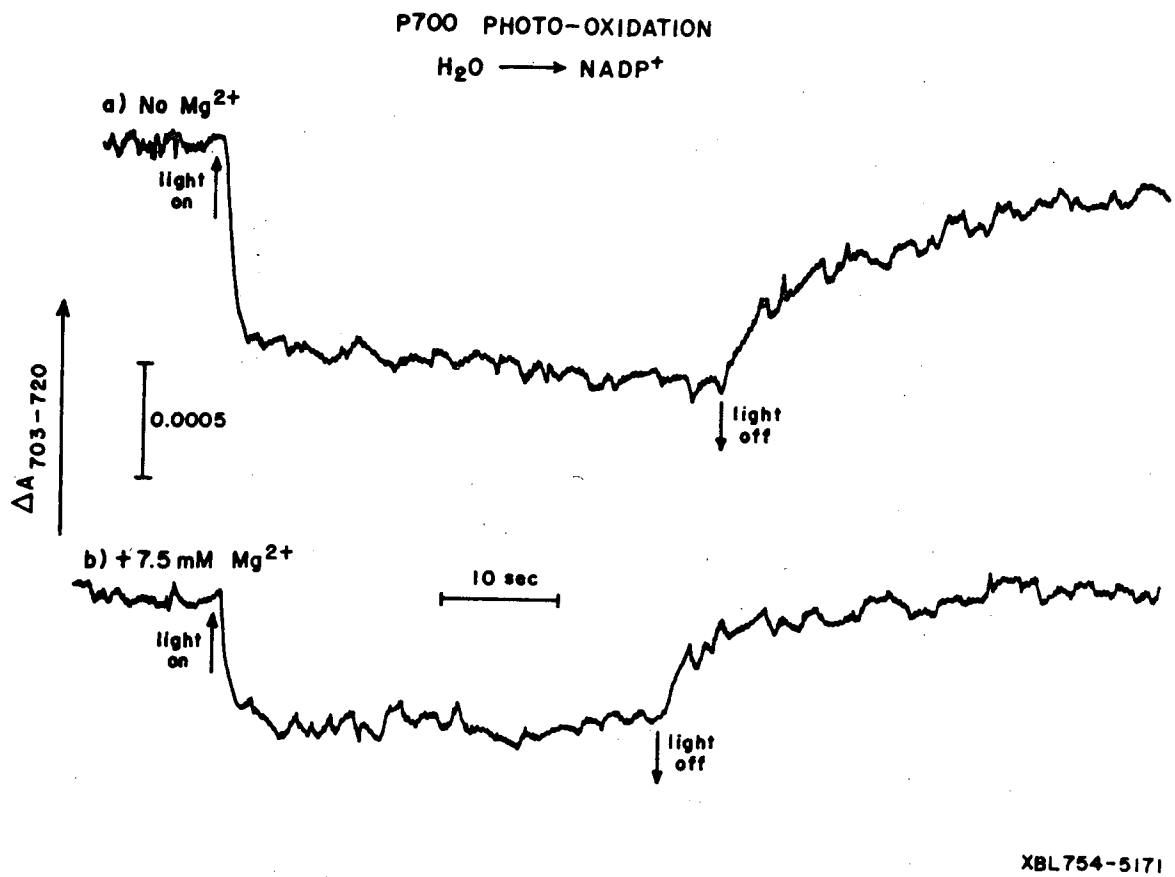

Figure IV.4 Effect of Mg^{2+} on P700 photo-oxidation in the $H_2O \rightarrow NADP^+$ system. Illumination conditions as in Figure IV.2; Fd concentration, 40 μg/ml; $NADP^+$ concentration, 0.6 mM. Fresh dark adapted samples were used in both a) and b).

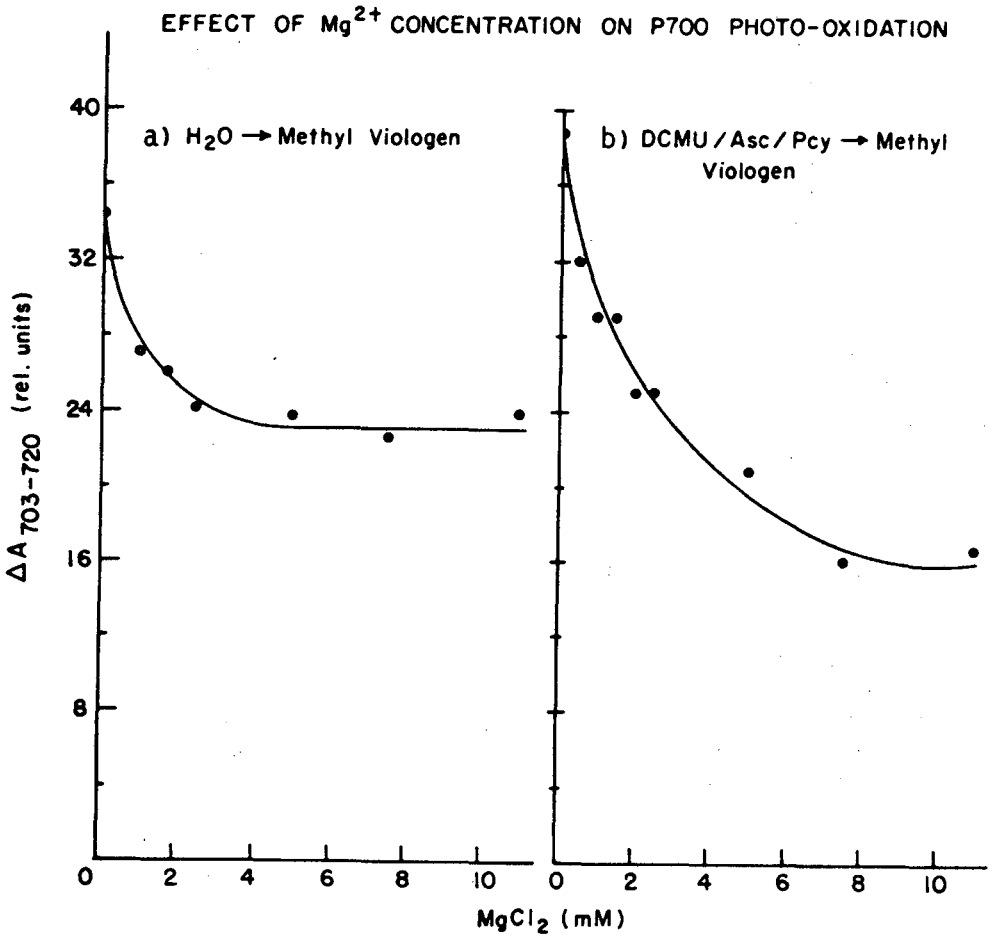

Figure IV.5a Effect of Mg^{2+} concentration on P700 photo-oxidation in the H_2O → methyl viologen system. Illumination conditions as in Figure IV.2; methyl viologen concentration, 0.1 mM. Fresh dark-adapted samples were used at each Mg^{2+} concentration shown on the figure.

Figure IV.5b Effect of Mg^{2+} concentration on P700 photo-oxidation in the DCMU/Asc/Pcy → methyl viologen system. DCMU concentration, 0.01 mM; Asc concentration, 2mM; Pcy, saturating amount; methyl viologen, 0.1 mM; other conditions as described in Figure IV.5a.

B. Systems with Non-physiological Electron Donor Systems: The studies of the effect of Mg^{2+} on the P700 photobleaching in the $H_2O \rightarrow$ methyl viologen (or $Fd/NADP^+$) system are rather complicated to interpret, because we must consider Mg^{2+} effects on PS II energy utilization and electron transfer in addition to possible Mg^{2+} interactions with PS I. Therefore, we have also studied how Mg^{2+} affects P700 photobleaching in DCMU poisoned chloroplasts, where we need not be concerned about possible effects of Mg^{2+} on PS II electron flow. In these studies of DCMU poisoned samples we have primarily relied on the artificial electron donor couple Asc/Pcy. Figure IV.6 shows for the DCMU/Asc/Pcy $\rightarrow$ methyl viologen system that addition of 7.5 mM Mg^{2+} causes a marked ($\sim$55%) decrease in the extent of P700 photobleaching. Similar results were obtained for the DCMU/Asc/Pcy $\rightarrow$ $Fd/NADP^+$ system (Figure IV.7). The concentration dependence of the Mg^{2+} induced quenching of the P700 photobleaching for the DCMU/Asc/Pcy $\rightarrow$ methyl viologen system is shown in Figure IV.5b. We observe that maximal quenching of P700 photobleaching occurs at a Mg^{2+} concentration of about 7.5 mM.

C. Effects of Mg^{2+} on the Rate of $NADP^+$ Reduction: Figure IV.8 shows that under fairly high actinic intensities (250 $\mu w/cm^2$ 7.5 mM Mg^{2+} causes a significant (100%) increase in the rate of the $H_2O \rightarrow NADP^+$ reaction. The effect of 7.5 mM Mg^{2+} on the rate of $NADP^+$ reduction in the DCMU/Asc/Pcy $\rightarrow$ $Fd/NADP^+$ reaction is shown in Figure IV.9. We observe that Mg^{2+} markedly stimulates ($\sim$6 fold) the rate of $NADP^+$ reduction in this system.

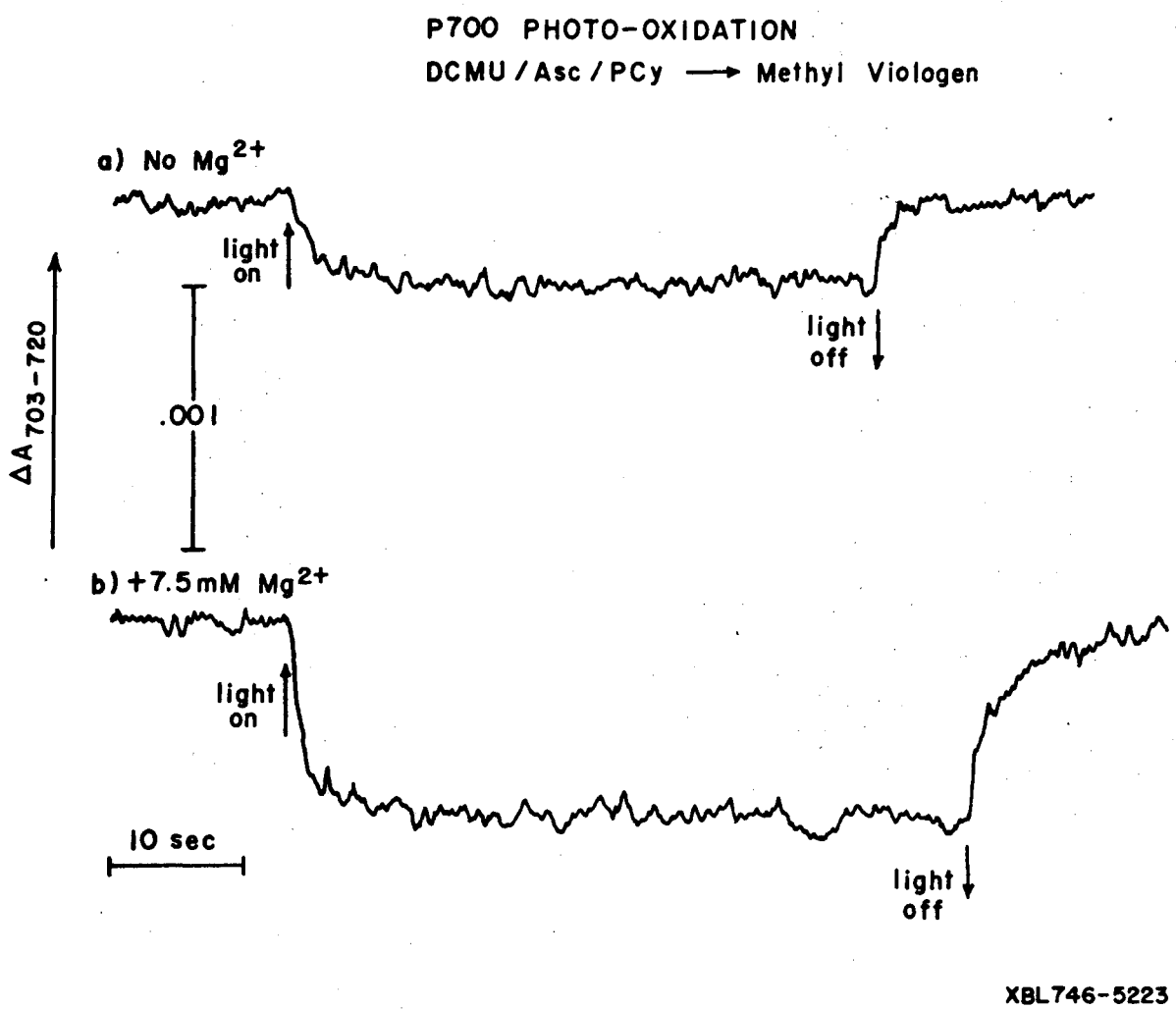

Figure IV.6 Effect of Mg^{2+} on P700 photo-oxidation in the DCMU/Asc/Pcy methyl viologen system. Illumination conditions as Figure IV.2. DCMU concentration, 0.01 mM; Asc concentration, 2mM; Pcy, saturating amount; methyl viologen concentration, 0.1 mM. Fresh dark-adapted samples were used in both a) and b).

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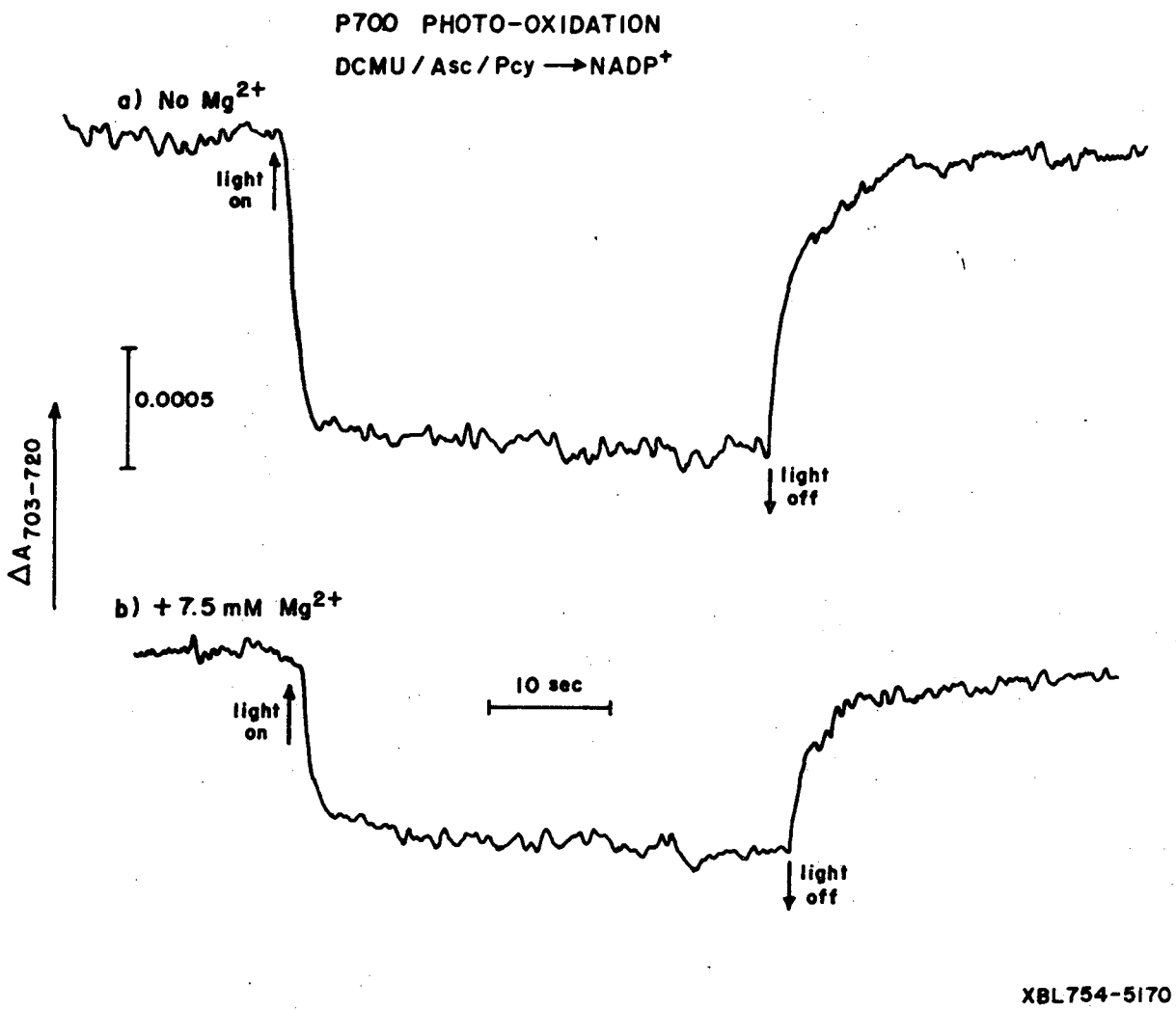

Figure IV.7. Effect of Mg^{2+} on P700 photo-oxidation in the DCMU/Asc/Pcy → $NADP^+$ system. Illumination conditions as in Figure IV.2; DCMU concentration, 0.01 mM. Asc concentration, 2 mM, Pcy, saturating amount; Fd concentration, 40 μg/ml; $NADP^+$ concentration, 0.6 mM. Fresh dark-adapted samples were used in both a) and b).

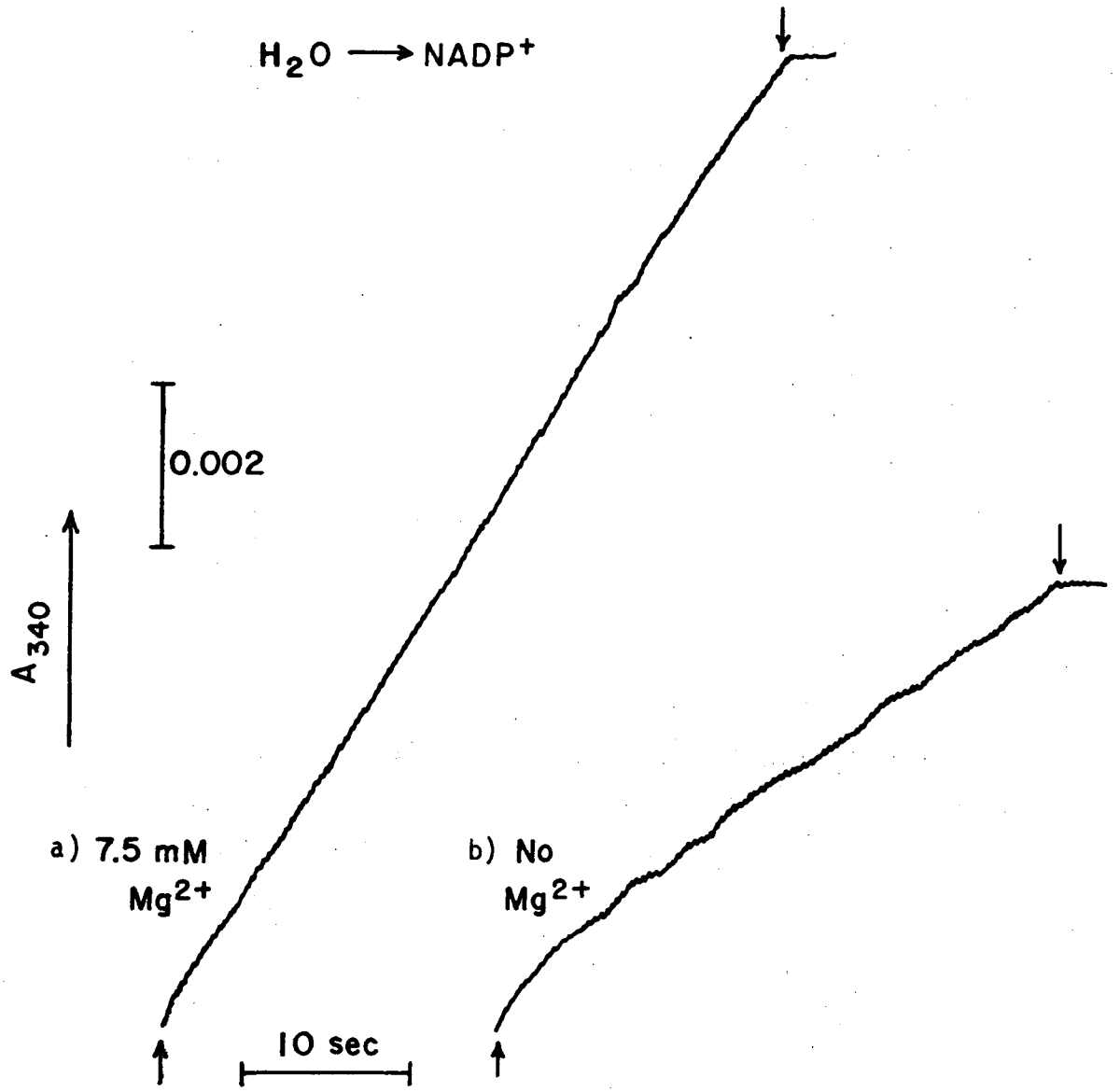

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Figure IV.8. Effect of Mg^{2+} on the rate of the $H_2O \rightarrow NADP^+$ reaction. Actinic wavelength, 430 nm; actinic intensity, 250 μw/cm^2; Fd concentration, 40 μg/ml; $NADP^+$ concentration, 0.6 mM. Fresh dark-adapted samples used for both a) and b). ↑ indicates start of actinic illumination; ↓ indicates end of actinic illumination.

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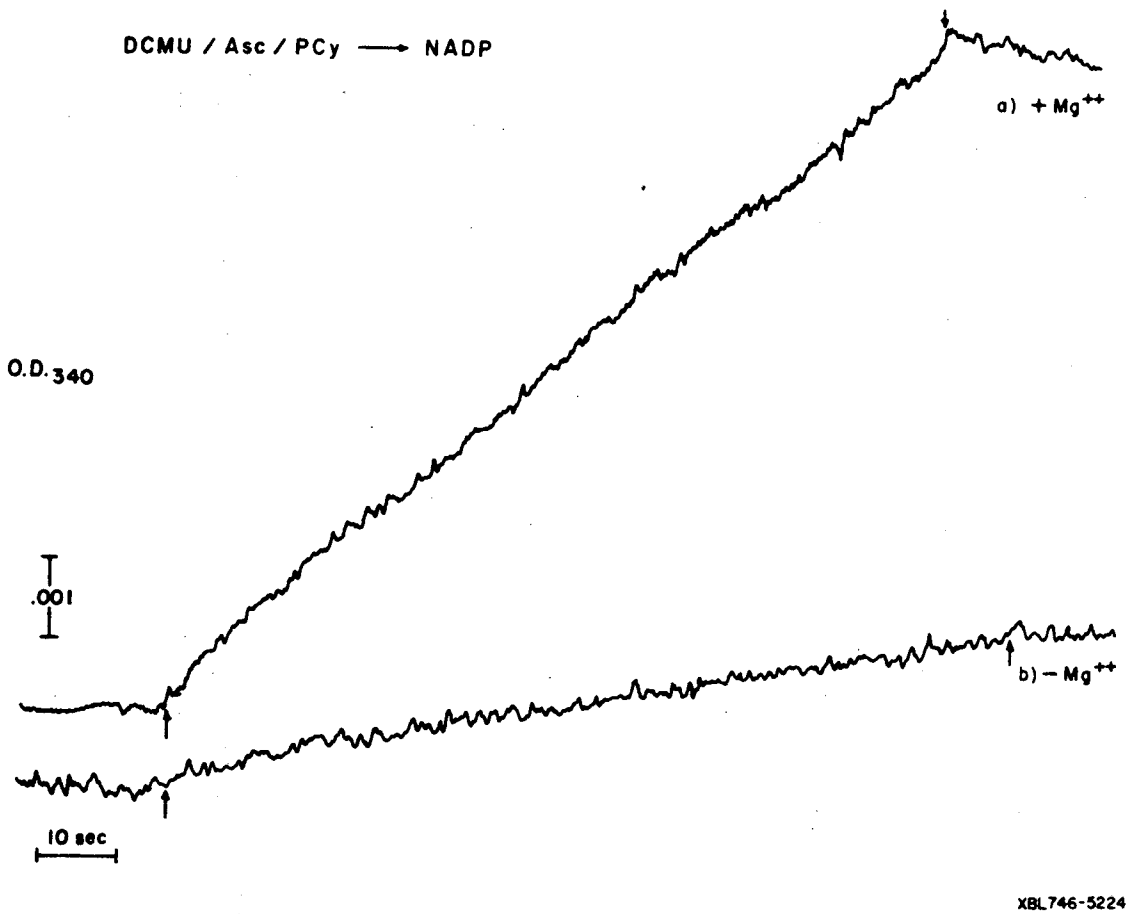

Figure IV.9 Effect of Mg^{2+} on the rate of the DCMU/Asc/Pcy → $NADP^{+}$ reaction. DCMU concentration, 0.01 mM; Asc concentration, 2mM; Pcy, saturating amount. Other conditions as in Figure IV.8.

<u>Discussion</u>: P700 can be photo-oxidized by the following process:

$$P700 \cdot X \xrightarrow{h\nu} P700^{+} \cdot X^{-} \qquad P700 = \text{PS I trap}$$

$$X = \text{PS I primary acceptor}$$

The photo-oxidized $P700^{+}$ can then be reduced to P700 via non-cyclic electron flow from the primary PS I electron donor to the oxidized trap:

$$P700^{+} + D \longrightarrow P700 + D^{+} \qquad D = \text{primary non-cyclic electron donor to PS I}$$

(The oxidized form of the donor D^{+} is eventually reduced via non-cyclic electron flow from H_2O or an artificial donor.) The photo-oxidized $P700^{+}$ can also be reduced by a direct back reaction, $P700^{+} \cdot X^{-} \rightarrow P700 \cdot X$, or via a cyclic electron pathway:

$$P700^{+} \cdot X^{-} + C \rightarrow P700^{+}\ X + C^{-} \qquad C = \text{PS I cyclic electron carrier}$$

$$P700^{+} \cdot X + C^{-} \rightarrow P700\ X + C$$

Although P700 does absorb at 703 nm, $P700^{+}$ does not absorb at this wavelength. Therefore, the extent of photobleaching at 703 nm provides a measure of the degree of photo-oxidation of P700. As seen in Figures IV.3, IV.4, IV.6, and IV.7, we observe Mg^{2+} quenching of P700 oxidation in the $H_2O \rightarrow$ methyl viologen, $H_2O \rightarrow Fd/NADP^{+}$, DCMU/Asc/Pcy $\rightarrow$ methyl viologen, and DCMU/Asc/Pcy $\rightarrow Fd/NADP^{+}$ reactions.

In our judgement, the most reasonable explanation for the Mg^{2+} quenching of P700 photo-oxidation is Mg^{2+} stimulation of non-cyclic electron flow. Consistent with this explanation, we also observe Mg^{2+} stimulation of $NADP^{+}$ reduction (Figures IV.8 and IV.9). Since we observe Mg^{2+} quenching with both the physiological (H_2O) and non-physiological (Asc/Pcy) donors and physiological ($Fd/NADP^{+}$) and

non-physiological (methyl viologen) acceptors, the most likely site of Mg^{2+} control of electron transport is between the site of Asc/Pcy donation to PS I and Y (the donor to Fd and methyl viologen).

One could also account for Mg^{2+} quenching of P700 photo-oxidation by one of the following explanations:

1. A Mg^{2+} induced decrease in excitation density at PS I trap,
2. Mg^{2+} stimulation of the back reaction $P700^{+}\cdot X^{-} \rightarrow P700\cdot X$,
3. Mg^{2+} inhibition of electron transport on the acceptor side of PS I,
4. A Mg^{2+} induced increase in the fraction of PS I electron flow which goes via a cyclic pathway.

However, all four of these explanations also require that Mg^{2+} decrease the rate of non-cyclic electron flow (measured as $NADP^{+}$ reduction). Since this requirement is incompatible with the Mg^{2+} stimulation of $NADP^{+}$ reduction seen in Figures IV.8 and IV.9, we feel our P700 results are most reasonably explained by Mg^{2+} stimulation of non-cyclic electron flow (Figure IV.10).

References for Chapter IV

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2. H. J. Rurainski, J. Randles, and G. E. Hoch, FEBS Letters 13, 98 (1971).
3. T. V. Marsho and B. Kok, Biochim. Biophys. Acta 333, 353 (1974).

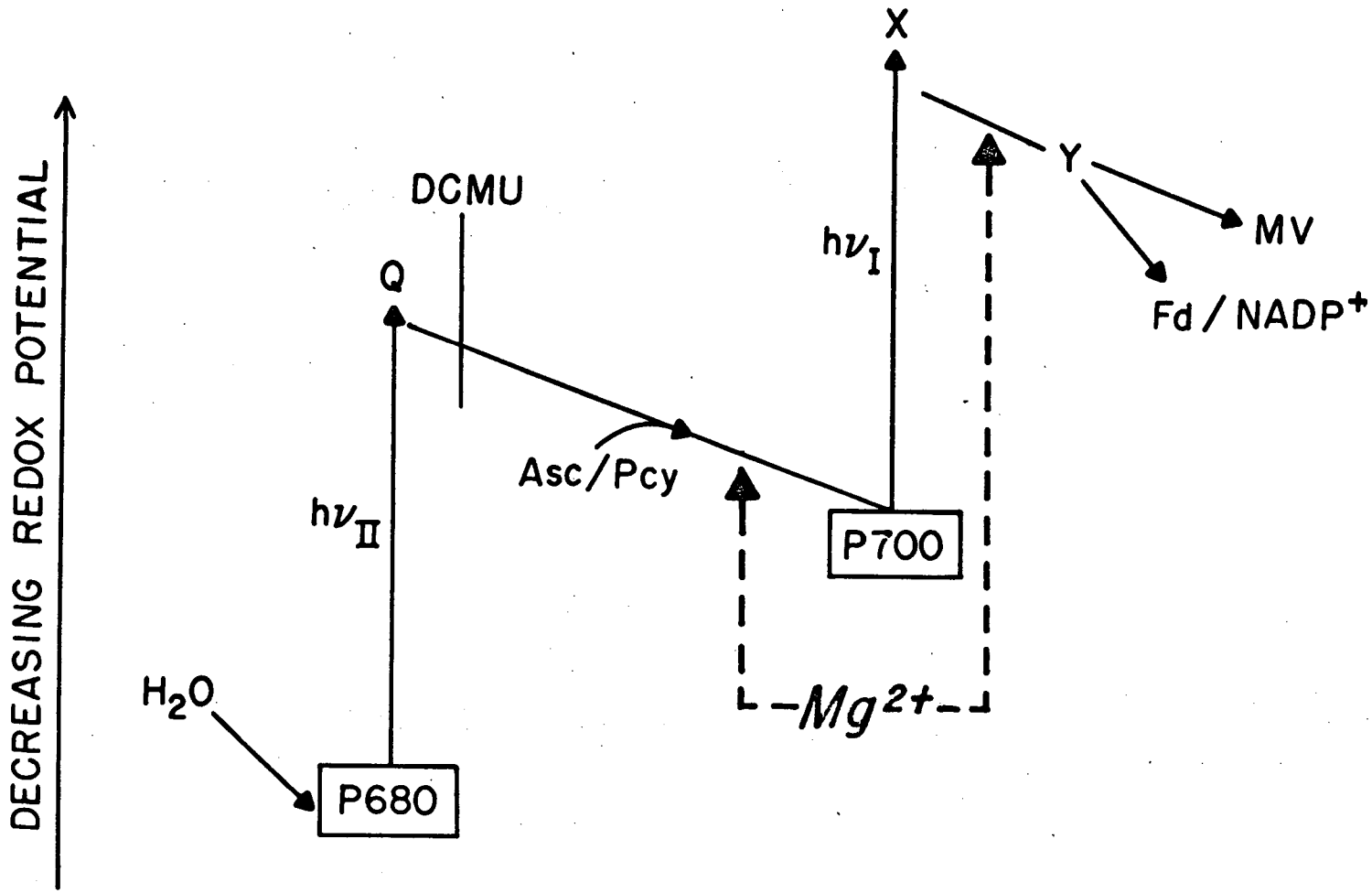

Figure IV.10. Possible sites of Mg^{2+} control of PS I electron transport. See text for detailed explanation.

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Chapter V

SUMMARY AND CONCLUDING REMARKS

At this point we would like to review the objectives of this thesis project, summarize our experimental results and conclusions, and suggest some areas for future research.

Motivation and Methodology: The goal of the research discussed in this thesis was to 1) identify and characterize those primary photosynthetic processes that are affected by Mg^{2+} and 2) explain how Mg^{2+} causes these changes in chloroplast function. As discussed in the introductory chapter, some previous investigators had proposed that Mg^{2+} caused inhibition of energy transfer from PS II → PS I, while other investigators suggested that Mg^{2+} increased energy transfer from PS I → PS II (Table I.1). We were especially interested in pursuing experiments that would allow us to decide if either of these proposals was correct or whether new models for the role of Mg^{2+} were necessary. The possibility of Mg^{2+} control of enemgy transfer between PS II and PS I is of interest because changes in the amount of energy transfer between the photosystems can be related to changes in the orientation of PS II pigments relative to PS I pigments.[1]

As discussed in the previous chapters, we have observed several effects of Mg^{2+} on chloroplast fluorescence, photochemistry and electron transport. For example, Mg^{2+} increases both the live and dead components of fluorescence. However, the degree of Mg^{2+} stimulation and the concentration of Mg^{2+} required to achieve maximum stimulation of

fluorescence differs for the two components of fluorescence. As another example, Mg^{2+} elevates the light-limited rate of PS I photochemistry when $NADP^+$ is the terminal acceptor but decreases the rate when methyl viologen is the terminal acceptor. These experiments, as well as others discussed in previous chapters, suggest that Mg^{2+} affects chloroplast photo-processes at more than one site. Hence it is probably an unrealistic oversimplification to explain all Mg^{2+} effects on the basis of a single mechanism, such as Mg^{2+} control of energy transfer from PS II → PS I. Therefore we have carried out independent investigations of the effect of the presence and absence of Mg^{2+} on a) chloroplast fluorescence, b) light limited rate of the H_2O → DCIP reaction, c) light-limited rates of the DCMU/Asc/TMPD → methyl viologen and DCMU/Asc/TMPD → $NADP^+$ reactions, d) P700 photo-oxidation and $NADP^+$ reduction under high light intensities. Chloroplast fluorescence and light-limited DCIP reduction are associated with PS II photochemistry. Hence measurement of the effect of Mg^{2+} on these observables yields information about the effect of Mg^{2+} on the primary photoprocesses associated with PS II. DCMU/Asc/TMPD → methyl viologen (and $NADP^+$) are reactions which involve PS I only. Therefore measurements of the effect of Mg^{2+} on the light limited rates of these reactions gives information about Mg^{2+} effects on PS I photo processes. P700 photo-oxidation and $NADP^+$ reduction are associated with PS I electron transport, hence by measuring the effect of Mg^{2+} on these observables we gain information about the effect of Mg^{2+} on PS I electron transport. As discussed in chapter II and chapter IV, special equipment modification was required for fluorescence and P700 measurements. After analyzing each of the Mg^{2+} effects according to the models presented in the text, we have

tried to consider what relationships, if any, should exist among these various measurements of Mg^{2+} effects. For example, since chloroplast fluorescence and DCIP reduction are associated with PS II photoprocesses we might expect to find a relationship between Mg^{2+} elevation of chloroplast fluorescence and Mg^{2+} elevation of the light-limited rate of DCIP reduction. Similarly, if Mg^{2+} affected energy distribution between the photosystems we might expect to find a relationship between Mg^{2+} effects on light-limited PS II photochemistry and PS I photochemistry.

Experimental Results and Conclusions: To account for the observation that Mg^{2+} stimulation of live fluorescence has a different concentration dependence than Mg^{2+} stimulation of dead fluorescence (Fig. II.3), we have proposed two models for the arrangement and function of PS II pigment arrays associated with chloroplast fluorescence. According to one model, the two site model (Fig. II.8), live fluorescence is both sensitized and emitted by a different pigment array than dead fluorescence. In the other model, the single site model (Fig. II.9), separate live and dead pigment arrays sensitize the two components of emission which come from a common fluorescent pigment array. According to both models the live pigment array sensitizes both live fluorescence and PS II photochemistry while the function of the dead pigment array is still not known. At low actinic intensities, we observe a direct proportionality between live fluorescence and actinic intensity for samples with and without Mg^{2+}. Under these conditions the live fluorescence level of Mg^{2+}-containing samples is twice as large as the level for samples without Mg^{2+} (Fig. II.5). In DCMU and dithionite poisoned samples we find Mg^{2+} approximately doubles the level of total fluorescence (Fig. II.7 and Table II.1). Analysis of

these results led to the conclusion that Mg^{2+} stimulation of live fluorescence results from a Mg^{2+} induced increase in the effective size (absorption cross-section) of the PS II live pigment array. The Mg^{2+}-elevated increases in the light limited rate of DCIP reduction (Fig. III.2) are, within the limits of experimental error, about the same as the Mg^{2+} induced increases in live fluorescence. Using the kinetic model for PS II photochemistry presented in chapter II, we have concluded that Mg^{2+} elevation of DCIP reduction is also most simply explained by a Mg^{2+}-induced increase in the effective size of the PS II live pigment array. Our analysis of our fluorescence and DCIP data does not allow us to support the previous proposal that Mg^{2+} blocks energy transfer from PS II → PS I.

As discussed in chapter II and chapter III, a Mg^{2+}-induced increase in PS II live antenna size could involve a Mg^{2+}-induced "connection" of previously "disconnected" chlorophyll or a Mg^{2+}-induced spillover of energy from PS I → PS II. We had hoped to distinguish between these two suggested roles of Mg^{2+} action by studying the effect of Mg^{2+} on light-limited PS I photochemistry. However, we were unable to determine whether Mg^{2+} effects on PS II also involve PS I because we observed opposite effects of Mg^{2+} on PS I photochemistry with different electron acceptors (Fig. III.3 and Table III.1). Thus it is not yet clear whether Mg^{2+} effects on light-limited PS I photochemistry are related to Mg^{2+} effects on PS II photochemistry and fluorescence, or whether other factors, such as PS I cyclic electron flow, are of significance in the interpretation of these experiments. Clearly, additional experiments are needed to resolve these questions.

In addition to studying the effects of Mg^{2+} on energy utilization by the photosystems, we also explored the effect of Mg^{2+} on the photosynthetic electron transport chain. The Mg^{2+} quenching of P700 photooxidation (Figs. IV.3, IV.4, IV.6, IV.7) and Mg^{2+} elevation of $NADP^+$ reduction (Figs. IV.8 and IV.9) appear to indicate that Mg^{2+} increases the rate of non-cyclic electron flow. Studies with different electron acceptors and donors indicate that the site of Mg^{2+} control of electron transport is between the site of Asc/Pcy donation to PS I and Y (the electron donor to methyl viologen and ferredoxin) (Fig. IV.10).

Evaluation of Experiments and Future Experiments: Since Mg^{2+} is present in almost all chloroplast preparation mixtures it is clearly of importance to know how this divalent cation affects primary reactions in chloroplasts. Before we can study and understand the effects of complex reagents such as silicotungstic acid (believed to reverse DCMU unhibition of electron transport) and Antimycin A (believed to uncouple cyclic electron transport) it is first necessary to understand the effects of the basic components of our reaction mixture.

In the intact leaf there is a light-driven pumping of Mg^{2+} ions from the cytoplasm to the chloroplast.[2] This change in Mg^{2+} concentration is known to affect the ability of the chloroplast to carry out photosynthesis.[2] Studies of the type carried out in this thesis (with broken chloroplasts) may explain why this change in Mg^{2+} concentration alters the efficiency of photosynthesis in the intact leaf.

As a result of the experiments described in chapter II we have proposed two different kinetic models to explain chloroplast fluorescence (Figs. II.8 and II.9). In the future we hope to evaluate these models for chloroplast fluorescence through the use of fluorescence lifetime

measurements. A chloroplast fluorescence decay curve composed of a single component would tend to support the single site model and rule out the two site model. A chloroplast fluorescence decay curve which is found to be composed of two components would tend to support the two site model and exclude the single site model. If we find the fluorescence decay curve is composed of three or more components, it will be necessary for us to formulate new models for chloroplast fluorescence.

Measurement of Mg^{2+} effects on P700 photo-oxidation, $NADP^+$ reduction, and methyl viologen reduction (oxygen uptake) with different donors to PS I should enable us to further localize the site of Mg^{2+} control of non-cyclic electron flow on the photosynthetic redox carrier chain.

References for Chapter V

1. N. Murata, Biochim. Biophys. Acta 189, 171 (1969)
2. P. S. Nobel and D. C. Lin, Arch. Biochem. Biophys. 145, 622 (1971)

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Appendix

The simplest model that has been proposed to explain chloroplast fluorescence is one where there is a single type of PS II pigment array.[1] In this view, live and dead fluorescence are sensitized and emitted by the same array of chlorophyll pigments. In this interpretation dead fluorescence represents emission that arises during the course of energy migration from the pigment antenna to the PS II trap. Dead emission is therefore independent of the state of the PS II trap. In contrast, live fluorescence in this model, as in other models, represents emission which competes with PS II photochemistry as a relaxation pathway for excited chlorophyll in the pigment array containing the trap. We can thus represent the processes responsible for live and dead fluorescence with the following equations:

Process		Rate
Excitation:	$Chl + h\nu \rightarrow Chl^*$	σI_m
Trapping and photochemistry:	$Chl^* + TA \xrightarrow[k_T]{} Chl + T^*A \rightarrow$ photochem.	$k_T(TA)(Chl^*)$
Fluorescence:	$Chl^* \xrightarrow[k_f]{} Chl + h\nu'$	$k_f(Chl^*)$
Thermal decay and spillover:	$Chl^* \xrightarrow[k_h]{} Chl +$ "heat"	$k_h(Chl^*)$

where Chl = ground state molecule in pigment array

Chl^* = excited singlet state molecule in pigment array

TA = ground state of open reaction center

T^*A = excited state of reaction center

k_T = rate constant for energy transfer from excited antenna pigment to trap

k_f = rate constant for de-excitation of excited antenna pigments by fluorescence

k_h = rate constant for de-excitation of excited antenna pigments by thermal losses and spillover to PS I

σ = effective absorption cross section (size) of PS II pigment array

I_m = intensity of modulated measuring beam

F_D = level of dead fluorescence

F_T = level of total fluorescence

F_L = level of live fluorescence

We can represent the three components of fluorescence by the following equations:

$$F_D = (\sigma\ I_m)\ /\ (k_T(TA)_0 + k_f + k_h)$$

$$F_T = (\sigma\ I_m)\ /\ (k_T(TA) + k_f + k_h)$$

$$F_L = F_T - F_D = (\sigma\ I_m)\ [(1/(k_T(TA) + k_f + k_h)] - 1/(k_T(TA)_0 + k_f + k_h)$$

where $(TA)_0$ = open trap concentration when <u>all</u> traps are in the open state.

Since $k_t(TA)_0$ is a constant which is independent of actinic intensity; F_D is of the same order of magnitude as F_T (Fig. II.2), and (TA) is a function of I_{act}, we find that in this model F_L is not a simple function of I_{act}. Consequently, we are unable to reconcile the direct proportionality between live fluorescence and actinic intensity seen in Figure II.5 with the functional form of this model. Assuming as in Chapter II that (TA) α $1/I_{act}$, this model predicts that F_T rather than F_L should be directly proportional to I_{act}. This prediction is clearly in conflict with the data of Figure II.5. If the approximation $F_T \approx F_L$ were valid, we could reconcile this model with our data. However, since F_D represents a substantial fraction of F_T in these studies, this approximation cannot be used.

References for Appendix

1. W. L. Butler and M. Kitajima, Biochim. Biophys. Acta 376, 116 (1975).

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