INDUCED AND NATURAL DELAYED LUMINESCENCE IN GREEN PLANT PHOTOSYNTHESIS

By

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ABSTRACT

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We have investigated the effect of an externally applied electric field on preilluminated chloroplasts in order to obtain information regarding the structure of the photosynthetic apparatus. A major manifestation of the externally applied field is the enhancement of delayed luminescence (d.l.) Delayed luminescence originates from a reversal of photochemical events, specifically, the events associated with Photosystem II (PSII). Therefore, the enhanced luminescence is ascribed to a field induced destabilization of intermediate states of PSII.

In order to characterize the electric field effect, nonperturbed d.l. (in the 7 to 200 μs time range) was examined for both fully functional chloroplasts and chloroplasts which had been inhibited in a controlled and well characterized manner. In spite of the significant perturbation of PSII, d.l. was nearly

identical for both untreated and inhibited samples and displayed pH dependent, biphasic kinetic behavior. In each case, we ascribe the fast phase of d.l. to the reduction of the oxidized form of the PSII reaction center ($P680^+$) by Z in units with inhibited electron transport on the oxidizing side of PSII. For untreated chloroplasts, this phase is probably a reflection of damaged centers. The slower phase is ascribed to a heterogeneity in P680. A number of theoretical models are investigated in order to describe the d.l. phenomena observed. By using a recombination model, modified to include oxidized reaction center quenching effects, a number of experimental observations can be rationalized.

In contrast to natural delayed light emission, the field induced luminescence is sensitive to physiological PSII events. We are able to ascribe much of the EPL effect to specific events associated with the oxidizing side of PSII. Based on these experiments, we are able to postulate relative orientations within the photosynthetic membrane of various PSII components.

The first man I saw was of a meager aspect, with sooty hands and face, his hair and beard long, ragged and singed in several places. His clothes, shirt, and skin were all of the same colour. He had been eight years upon a project for extracting sunbeams from cucumbers, which were to be put into vials hermatically sealed and let out to warm the air in raw inclement summers. He told me he did not doubt in eight years more that he should be able to supply the Governor's gardens with sunshine at a reasonable rate; but he complained that his stock was low, and entreated me to give him something as an encouragement to ingenuity, especially since this had been a very dear season for cucumbers. I made a small present, for my lord had furnished me with money on purpose, because he knew their practice of begging from all who go to see them.

From Jonathan Swift: Travels into Several Remote Nations of the World... by Captain Lemuel Gulliver

ACKNOWLEDGMENTS

I acknowledge the assistance of the departmental electronic and machine shops in the design and construction of the experimental apparatus described here. I would also like to thank all those people, who through their friendship and encouragement, greatly facilitated completion of this work.

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INTRODUCTION

Photosynthesis is a multifaceted and complex phenomenon. The initial phase of photosynthesis, the absorption of light and subsequent photooxidation of a specialized pigment, occurs within the thylaklid membrane. There is an increasing awareness of the importance of the organization within the membrane of the various components involved with this process. This includes not only the orientation of the photooxidizable pigment relative to the antenna system to assure efficient energy transfer, but also the relative orientation of the electron donors and acceptors to assure high chemical yields. Investigations into this aspect of photosynthesis are limited because of the lack of specific probes. It was recently reported that the application of an external electric field on preilluminated chloroplasts results in an enhanced delayed luminescence [1,2]. Delayed luminescence originates from a chemically generated exciton which is formed as a result of a reversal of the light induced electron transfer reactions. Thus, the enhanced luminescence was ascribed to a field induced destabilization of the photochemical products. Because of the directional nature of the electric field,

this technique potentially represents a sensitive and convenient probe to the relative orientation of the delayed luminescence precursors. The purpose of this work is to investigate and characterize the usefulness of the electric field technique as a probe of photosynthesis. In addition, a critical analysis of the limitations of delayed luminescence as an indicator of the electron transfer reactions of photosynthesis will be presented.

A. Overview of Photosynthesis

Green plant photosynthesis, a complex phenomenon representing the transformation of solar or light energy into chemical energy, occurs in subcellular organelles known as chloroplasts. Structurally, chloroplasts are typically 1 to 10 microns in diameter and consist of an easily sheared outer membrane and a continuous inner membrane. This inner membrane regularly forms flattened vesicles about 0.5 microns in diameter known as thylakoids; the thylakoids are arranged in stacked configurations called grana and are interconnected by regions of unstacked membranes referred to as the intergranal lamellae. Many of the reactions of photosynthesis are associated with the thylakoid membrane. Surrounding the internal components of the chloroplasts is the matrix or stroma. During standard isolation procedures, the outer membrane is sheared and only the thylakoids are obtained; these are formally known as class II chloroplasts. In the following

discussion, the terms class II chloroplasts, thylakoids, and chloroplasts will be used synonymously unless noted otherwise. Class I chloroplasts, which have the outer membrane intact and which may be obtained through a mild isolation procedure [3], were not involved in any of the experiments described here.

The overall equation for photosynthesis may be expressed as

$$6CO_2 + 6H_2O \longrightarrow C_6H_{12}O_6 + 6O_2$$

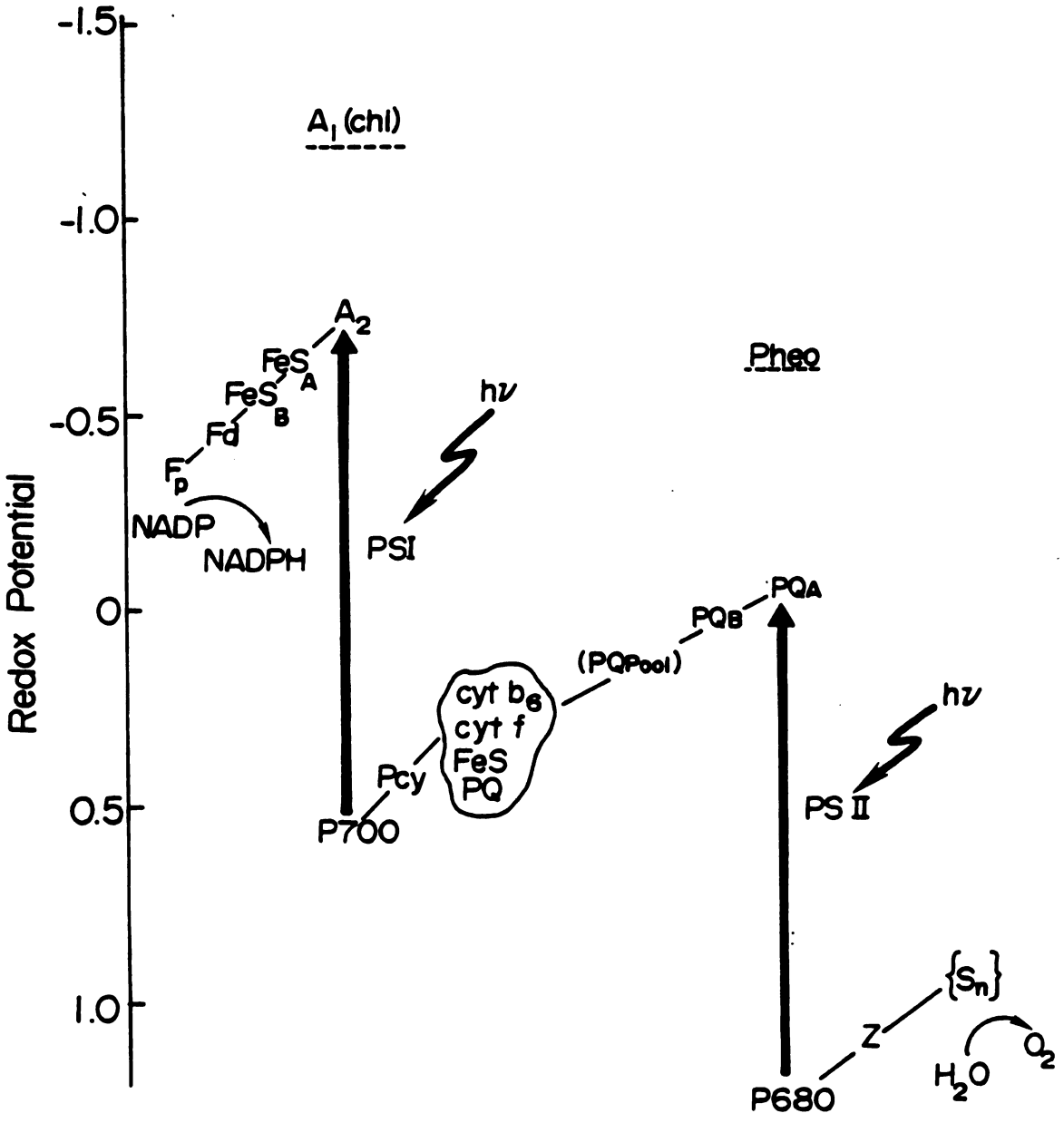
where $C_6H_{12}O_6$ represents a sugar molecule. This equation is an oversimplification of the complexity of photosynthesis, and is in fact misleading. Traditionally, photosynthesis has been partitioned into two aspects, the light reactions and the dark reactions. In the dark reactions, the chemical energy that is stored in the products of the light reactions, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine 5'-triphosphate (ATP), is utilized in the reduction of CO_2 to carbohydrate by a process referred to as either the reductive pentose cycle or the Calvin cycle. This process is carried out in the stroma primarily by water soluble, nonmembrane associated proteins. Calvin and coworkers [4] did the initial characterization of the reactions which comprise this cycle.

The model which most concisely and adequately describes the light reactions is known as the Z-scheme (Fig. I-1). In the Z-scheme each constituent of the photosynthetic

FIGURE I-1

The Z scheme. The model for the photosynthetic light reactions and energetics. Abbreviations: Sn, the oxygen evolving complex; Z, donor to P680; P680, the reaction center of PSII; Pheo, pheophytin; PQ_A, the primary stable quinone acceptor of PSII, PQ_B, the secondary quinone acceptor of PSII; PQ pool, the plastoquinone pool; cyt b6, cytochrome b6; cyt f, cytochrome f; FeS, iron sulfur center; PQ, a plastoquinone species; Pcy, plastocyanin; P700, the reaction center of PSI; A1, the primary acceptor of PSI; A2, the secondary acceptor of PSI; FeS_A and FeS_B, iron sulfur centers a and b; Fd, ferredoxin; Fp, ferredoxin-NADP reductase; NADP, nicotinamide adenine dinucleotide phosphate. Details explained in the text.

Photosynthetic Light Reactions and Energetics
Idealized Scheme



electron transport chain associated with the light reactions is plotted in sequential order and relative to its energetics. An examination of the Z-scheme shows that electrons are transferred "uphill" from water with an average reduction potential of +815 mV at pH 7.0 to NADPH at a reduction potential of -350 mV. This process is mediated by the photooxidation of two specialized chlorophylls or reaction centers which act in series. The existence of two independent photoreactions leads to the concept of two photosystems, Photosystem I (PSI), which is associated with NADP reduction, and Photosystem II (PSII), which is associated with the oxidation of water. P700 and P680 are the reactions centers, or primary electron donors, of PSI and PSII respectively. The origin of the name for the reaction centers arises from the photobleaching in the optical absorption spectrum, centered around either 700 or 680 nm, which is observed when PSI or PSII, respectively, undergo photochemistry. Thus, far red light (λ=700 nm) is preferentially absorbed and used by PSI.

Although it cannot be completely resolved, the optical difference spectrum (P700 — $P700^+$) displays two negative bands centered around 703 and 690 nm respectively and a broad positive band around 820 nm [5]. The band around 820 nm is attributed to the chlorophyll cation formed upon photooxidation of P700. The negative doublet may be indicative that P700 is a dimer of chlorophyll a (chl a). The dimer model of P700 is also supported by the ESR signal

attributed to $P700^+$ which has a g value of 2.0025 and a linewidth of 7 gauss, whereas in solution the chl a cation has a linewidth of 9 gauss. The narrowing of the linewidth was originally ascribed to a delocalization of spins over two identical chlorophyll molecules [6]. The ESR signal for $P680^+$ observed at low temperature (g=2.002) has a bandwidth of 7 to 8 gauss, which provides evidence that P680 is also a dimer [7]. Model studies by Davis et al [8] have, however, demonstrated that the ESR lineshape may be due to special environmental effects. This raises some doubts regarding the dimer model for P680 and P700. Moreover, Wasielewski et al. [9] pointed out that the narrower linewidth of the ESR signal for $P700^+$ relative to that observed in solution for the chl a^+ may arise from a difference in the unpaired electron spin density distribution over a monomer. By cultivating the green alga *Scenedesmus obliquus* in a growth medium enriched in ^{13}C and ^{2}H, P700 and chl a is produced with ^{13}C in the porphyrin ring; and in an ESR experiment, the total spin density within the ring can be monitored. Under these conditions, the $P700^+$ ESR signal was comparable to the signal obtained for the *in vitro* chl a^+ monomer of similar isotopic composition.

P700 and P680 differ significantly in their respective reduction potentials. The reduction potential of P700 has been measured by direct titration with the reported value being approximately +490 mV [10]. Alternatively, the

potential of P680, which must generate an oxidant greater than +800 mV in order to be capable of oxidizing water, has not been directly measured. By using the measured midpoint potential for the electron acceptor of P680 (a pheophytin) of -610 mV coupled with the activation energy of 80 mV for the back reaction and the total light energy input, Klimov et al. calculated the E° of P680 to be around 1.14 V [11].

The concentration of reaction centers represents only about 0.25% of the total chlorophyll present. The role of the bulk chlorophyll and other pigments such as carotenoids is to serve as antenna; that is, light absorbed by the bulk pigments is transferred to the reaction center by a Förster type mechanism [12]. A quantum yield of essentially unity [13] is a testament to the efficiency of exciton transfer in photosynthesis. As a result of the large absorbance associated with the bulk pigments, optical studies on the reaction centers are difficult.

Upon excitation and subsequent photooxidation of the reaction center, a rapid electron migration through a series of intermediate carriers takes place. Though far from resolved, there appear to be some similarities on the acceptor side of P700 and P680. The emerging model is based upon the bacterial reaction center, P870, which, unlike P700 and P680, may be obtained in purified preparations [14]. In photosynthetic bacteria, electron transfer involves a bacteriochlorophyll dimer as the reaction center, a

bacteriochlorophyll mónomer (bchl) as a primary acceptor, a bacteriopheophytin (bpheo) as a secondary acceptor, and an ubiquinone (UQ_A) as a tertiary acceptor. As a result of the close proximity of an iron atom, the chemical properties of the quinone acceptor are significantly different from those that would normally be observed in solution [15]. The kinetics of electron transfer involving the primary and secondary acceptor are fast and have not been directly measured. The reduction time of the quinone is less than 200 ps [16]. The lifetime of the oxidized form of the tertiary acceptor is from several tenths to tens of ms and is often referred to as the primary stable electron acceptor of P870. The primary stable acceptor of P700 is P430, which appears to be an iron-sulfur protein capable of a two electron reduction. P430 is probably identical to the iron sulfur centers (FeS_A and FeS_B) detected by low temperature ESR studies [17]. The reported midpoint potentials were -553 mV and -594 mV. Under anaerobic conditions in the presence of dithionite to maintain P430 in the reduced state, P700 is still photooxidized and can be monitored by the ESR Signal I which has been attributed to $P700^+$. This observation indicates that a lower potential acceptor functions prior to P430. In experiments involving PSI particles lacking efficient System I reductants, $P700^+$ decayed with a halflife of about 250 μs when generated under reducing conditions. The decay is attributed to a back reaction between the reaction center and a lower

potential electron acceptor than P430. As the reduction potential was poised to a lower value by the addition of neutral red, the rate of the back reaction increased to a half-life of about 5 μs [18]. Thus, sandwiched between P700 and P430, there appear to be two acceptors, A1 and A2. The chemical nature of A1 and A2 is uncertain, but based upon its reduction potential and the chemical composition of PSI particles, it has been suggested that A1 is a chlorophyll monomer [19]. A2 is probably identical to the species X described by Dismukes and Sauer [20], who argued that X was a bound iron-sulfur center.

In PSII, the analogy with photosynthetic bacteria is even closer. In photosynthetic bacteria, a triplet state having a non-Boltzmann spin distribution was chemically generated for P870 at low temperature. This spin polarized triplet resulted from the back reaction between $P870^+$ and $bpheo^-$ [21]. Subsequent investigations demonstrated the same phenomenon for PSII. Since there is no evidence for the role of a chlorophyll monomer in PSII electron transport, this indicates that the primary acceptor of PSII is a pheophytin [22]. As in the case of bacteria, the primary stable acceptor is a quinone. In this case, it is a plastoquinone species (PQ_A). The present model for the reaction centers of photosynthetic bacteria, PSI, and PSII may be summarized as

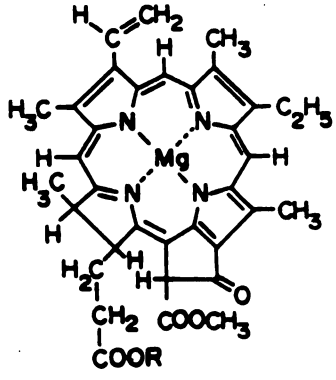
Bacteria:	$(BChl)_2$ P870	(BChl)	(BPheo)	$(UQ_A\text{-}Fe\text{-}UQ_B)$
PSII:	(CHl) P680		(Pheo)	$(PQ_A\text{-}Fe\text{-}PQ_B)$
PSI:	(Chl) P700	(Chl) A1	(FeS) A2	$(FeS_A\text{-}FeS_B)$ P430

In both photosynthetic bacteria and PSII there exists a second quinone that serves as an oxidant for the primary stable acceptor. The chemistry of both these quinone species is significantly affected by the interaction with a nearby iron atom. Structures for chlorophyll, pheophytin, plastoquinone, and the bacterial system analogs are shown in Figure I-2.

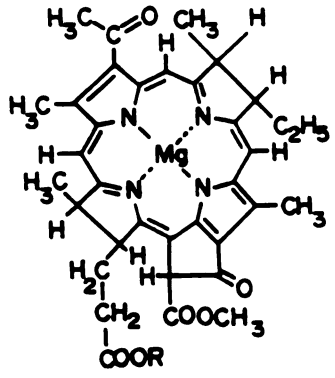
The role of the intermediate electron carriers on the acceptor side of the reaction center is to stabilize the light induced charge separation. The photochemical yield of photosynthesis is essentially unity. To achieve this high chemical yield, there is a significant loss of free energy. The potential difference between P700 and P430 is 1.1 V; this represents only about 60% of the available energy associated with a 700 nm photon (1.77 eV). In PSII, the difference in potential between P680 and PQ_A is about 1.20 V, or about 65% of the available energy associated with a 680 nm photon (1.85 eV). Since theoretical studies [23] indicate that in photosynthesis the maximal efficiency for the conversion of light energy to chemical energy is approximately 0.70, the actual yield for energy transduction is within about 90% of the hypothetical limit.

FIGURE I-2

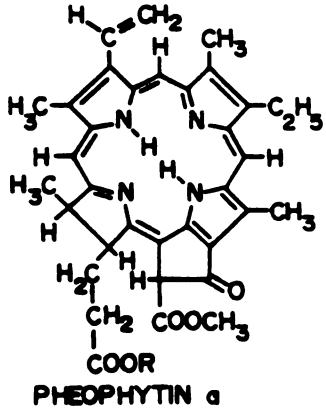
Molecular structures of chlorophyll a, pheophytin a, bacteriochlorophyll, bacteriopheophytin, plastoquinone and ubiquinone.



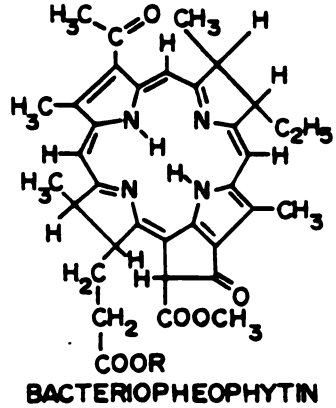
CHLOROPHYLL a



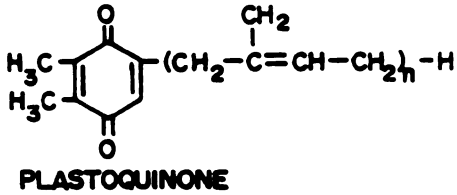
BACTERIOCHLOROPHYLL a



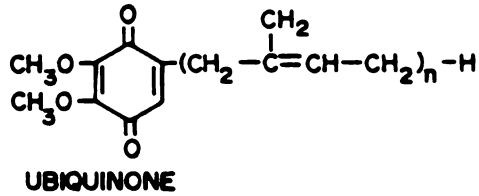
PHEOPHYTIN a



BACTERIOPHEOPHYTIN



PLASTOQUINONE



UBIQUINONE

Following P430 reduction in PSI, there is a series of electron transfer reactions involving P430, ferredoxin, ferredoxin-NADP reductase, and NADP. NADP supplies reducing equivalents for the reductive pentose cycle. Connecting P680 and P700 is an intersystem chain of electron carriers. Although the primary stable acceptor of PSII, PQ_A, is a quinone, which in principle can undergo a two electron reduction, it is only a single electron acceptor, and under physiological conditions the semiquinone anion is formed. However, because of the presence of an iron atom, the ESR signal which should be associated with a semiquinone anion becomes broadened at room temperature. As a result, it is detectable only at low temperatures in reaction centers of photosynthetic bacteria [15] and was only recently observed at low temperatures in PSII particles [24]. In bacteria, the extraction of the iron atom allows UQ_A to become fully reduced by the normal pH dependent quinone reactions [25]; the iron atom apparently lowers the second reduction potential of UQ_A to an inoperative level. However, structural effects associated with the extraction of the iron atom have not been completely ruled out.

Concomitant with the oxidation of PQ_A^- ($t_{1/2}$ = 100 to 600 μs) is the reduction of a second quinone species PQ_B. Unlike PQ_A, PQ_B is a two electron acceptor and is stable in the semiquinone form [26]. When fully reduced, PQ_B apparently exchanges with the plastoquinone pool (PQ pool). The PQ pool is an amalgamation of 7 to 10

plastoquinone molecules that serve as a reservoir for reducing equivalents produced by PSII. Following the PQ pool, there is a series of carriers that shuttle electrons to $P700^+$. These include cytochrome f (cyt f) and plastocyanin (Pcy). As the chemical species which directly reduces $P700^+$. Pcy serves as the secondary donor to PSI. A more detailed discussion of the intersystem chain may be found in a review by Avron [27].

The oxidizing side of PSII, that aspect of photosynthesis associated with water oxidation, has long been one of the least understood photosynthetic phenomena. A significant contribution towards the elucidation of the chemistry of oxgyen evolution was made in the classic experiments of Joliot et al. [28] and Kok et al. [29], in which the yield of evolved oxygen following a short actinic light pulse was measured. They observed that the yield of O_2 varied with flash number and displayed a damped oscillatory behavior of period four. The maximal yield occurred on the 3rd flash, with local maxima occurring on each subsequent fourth flash. To explain this, Kok postulated a phenomenological model invoking the existence of various S states, S_0, S_1, S_2, S_3, and S_4, in which the subscript refers to the number of oxidizing equivalents that are stored. Water oxidation is a four electron process ($2H_2O \rightarrow O_2 + 4H^+ + 4e^-$). The period four behavior suggests an independent chain model for electron transport on the oxidizing side of PSII. Thus a single PSII reaction center supplies oxidizing equivalents

to a specific oxygen evolving complex (OEC). The maximal yield following the third flash is attributed to a stable S_1 state, with only approximately 25% of the centers in the S_0 state. S_2 and S_3 relax to the S_1 state ($t_{1/2}$ = 1 to 10 s) [30], and S_4 reacts quickly with H_2O to form O_2 and S_0. The damped oscillations are due to multiple turnovers of PSII arising from the tail associated with a xenon flash lamp, as well as to misses which arise from centers which either do not undergo photochemistry or undergo photochemistry but deactivate in the dark. The four flash oscillatory behavior is often used to associate certain phenomena with PSII.

Between P680 and the OEC, there appears to be at least one intermediate electron carrier which is designated as either D [31] or Z [32]. The oxidized form of this intermediate may be monitored by the ESR Signal IIvf for intact O_2 evolving chloroplasts, and Signal IIf for chloroplasts with electron transport from the OEC inhibited [32]. Signal IIf and IIvf apparently arise from the same chemical species, but because of the rapid rate of reduction of Z^+ in intact O_2 evolving chloroplasts relative to that observed for chloroplasts with electron transport on the oxidizing side of PSII inhibited, IIf and IIvf display distinctly different decay kinetics.

The kinetics of the various reactions of PSII are complex and depend strongly upon pretreatments. PQ_A is reduced in less than 200 ps while the rate of electron

transfer through pheophytin is not directly measurable under physiological conditions [16]. The oxidation of PQ_A^- takes several hundred μs with reported times of 200 to 400 μs and 600 to 800 μs following odd and even flashes respectively [26]. The difference in kinetics is an indication of the effect of transforming PQ_B from the partially reduced semiquinone to the fully reduced state. Similar effects of oxidation state on electron transfer rate are observed for Z^+ reduction by the OEC; the rate of reduction of Z^+ ranges from <100 μs for the S_1 state to about 200 and 1000 μs for S_2 and S_3 states, respectively [33]. $P680^+$ reduction has numerous values reported in the literature. In dark adapted samples, Van Best et al. [34] reported a decay time of 30 ns. This rate slows down to 600 ns in the steady state [35]. Conjeaud et al. [36] mention that the $P680^+$ decay had a 7 to 10 μs and a 130 μs decay component. Although there exists a report in the literature indicating that the risetime of Signal IIvf, hence the rate at which Z is oxidized, is 15 μs [37], it is now apparent that the signal risetime was limited by interference from a CIDEP (chemically induced dynamic electron polarization) signal associated with PSI which arises from the forward reaction between $P700^+$ and X [38]. Thus, it is possible that Z is the direct donor to $P680^+$ in O_2 evolving chloroplasts, as it appears to be when water oxidation is inhibited (see below).

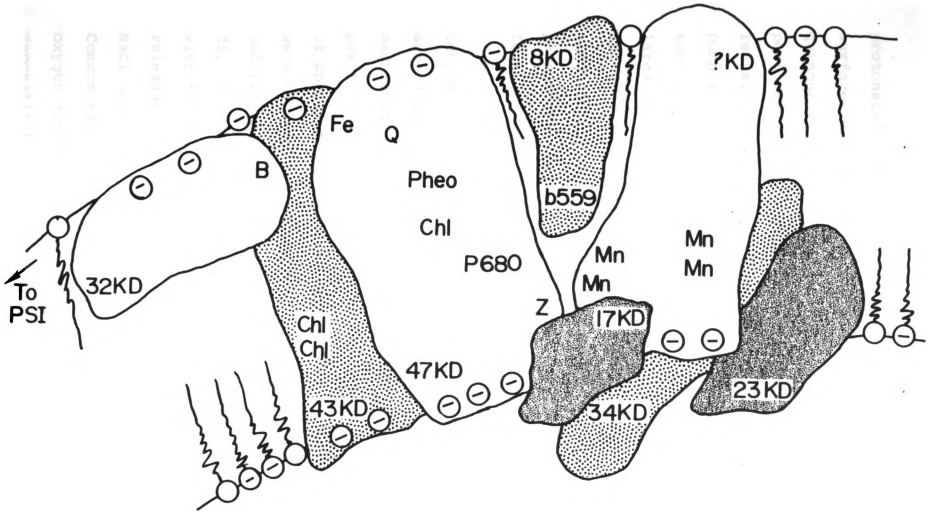
Electron transfer rates can be drastically affected by the use of inhibitors. DCMU completely blocks the $PQ_A \rightarrow PQ_B$ reaction. Treatments such as tris-washing inhibit the electron flow between Z and the OEC [39]. A secondary effect of tris-washing appears to be the decrease in the rate of reduction of $P680^+$ from submicroseconds to about 10 μs [31]. The actual $P680^+$ decay in dark adapted tris inhibited chloroplasts was reported to be biphasic with a dominant pH dependent phase, ranging from a half-life of 44 μs at pH 4.0 to a half-life of 3.5 μs at pH 8.0 and a minor pH independent phase with a halftime of 100 to 200 μs. The slower phase dominated the $P680^+$ decay on the second turnover indicating that there is only a one electron capacity in tris-washed chloroplasts. The pH dependence of $P680^+$ reduction was substantiated by parallel measurements on the decay of the ESR Signal II attributed to $P680^+$ and the rise of Signal IIf [40]. In PSII fragments with oxygen evolution activity inhibited by tris-washing, the kinetics of $P680^+$ decay and IIf rise were nearly identical to values reported in [31], thus directly verifying the role of Z as an electron donor to P680. PSII electron transport was recently reviewed by Bouges-Bocquet [41]. In order to account for the variable reduction rates of $P680^+$ (a submicrosecond and a 10 μs phase) and the ESR Signal IIvf decay, she argued for the existence of two electron carriers, Z_1 and Z_2 operating in parallel between

P680 and the OEC. The actual composition on the oxidizing side of P680 remains an area of active research.

The light reactions, unlike the dark reactions, are associated with membrane bound proteins; Figure I-3 shows postulated orientations for various components of PSII. Electron transport from primary donor (P680) to acceptor (Pheo, PQ_A) occurs vectorially (from inside to outside) across the membrane thus generating a light-induced transmembrane potential. One manifestation of the light induced membrane potential is an accompanying shift in the absorption bands of some pigments. The absorption change (light—dark) at 515 nm is attributed to such an electrochromatic shift of a carotenoid absorption band and is often used as an indicator for the relative magnitude of the membrane potential [42]. Electron transfer towards the outer surface was directly verified by Fowler and Kok [43]. By using nonsaturating light flashes, a potential difference was generated between two electrodes positioned at different levels in a chloroplast suspension. The electrode nearest the light source became negative. The actual distance separating P680 and PQ_A is not known. Some reports suggest that the initial charge separation spans the full membrane [36]; others argue that P680 and PQ_A are separated by a distance of about 10 to 20 Å or only about 25% of the total membrane thickness [44,45]. Although it is not shown in Figure I-3, the PQ pool apparently spans the full membrane. Reduction, which generates the

FIGURE I-3

Postulated orientation of various PSII components and proteins within the thylakoid membrane [50]. As indicated, the membrane surface charge probably arises from the lipid head groups as well as from the protein constituents of membrane. Z is situated towards the inner surface. PQ_A and PQ_B (labeled as Q and B) are depicted towards the outside surface of the membrane. (Details given in text).



PHOTOSYSTEM II

protonated hydroquinone species occurs towards the outer surface, whereas oxidation, which generates the deprotonated quinone form, is towards the inner surface. Thus the PQ pool serves not only as a reservoir for oxidation equivalents, but may also serve as a proton pump. This proton pump in conjunction with other proton releasing reactions such as water oxidation, can generate a pH gradient as large as 3 to 4 pH units ($pH_{[inside]} < pH_{[outside]}$) in the steady state [46]. This pH gradient and the accompanying electric field, according to the chemiosmotic model for phosphorylation [47], supply the free energy necessary to form ATP from ADP and Pi.

Figure I-3 also depicts the possible orientation and composition of various proteins correlated to PSII in accordance with the fluid mosaic model of biological membranes [48]. Bennoun et al. [49] reported that in particles enriched in PSII there were 3 integral proteins of approximate molecular weights 50, 47, and 3 KD which were closely associated with the reaction center. Additionally, 3 peripheral proteins of molecular weights 35, 21, 18 KD were reported to be less tightly associated with the reaction center [49]. Peripheral proteins released by washing inside-out chloroplasts with 250 mM NaCl had molecular weights of about 23 and 16 KD [50]. Concomitant with the salt washing was the inhibition of oxygen evolution. The oxygen activity was restored upon reconstitution of the 23 KD protein back into the membrane.

Metz et al. [51] noted the absence of a 34 KD protein subunit in a green algae mutant lacking the ability to oxidize water. Thus associated with the OEC there appear to be at least 3 protein subunits with approximate molecular weights of 17, 23, and 34 KD; the role of these polypeptides may possibly be structural in nature. It has been argued [49] that the 48 KD protein is associated with the reaction center; because of the high efficiency and the difficulty involved with inhibiting electron transport from P680 to PQ_A and from Z to P680, the PSII primary stable acceptor and secondary donor are assumed to be on the same protein moiety with the reaction center. Recent experiments involving various preparations of PSII particles support this hypothesis [52]. Other reports have shown that isolated subunits of molecular weight of 45 to 50 KD readily bind chlorophyll [53], thus this integral protein is probably part of the antenna complex of PSII. In a mutant of maize lacking the ability to carry out the secondary electron transport on the reducing side of PSII, a 34 KD subunit was missing [54]. Thus it is indicated in Figure I-3 that PQ_B is associated with this protein. Cytochrome b_{559} , a species that becomes photooxidized at low temperature but has no known physiological function [55], is depicted with an 8 KD protein in close proximity with P680. The protein immediately associated with the OEC has not been isolated, but because of the necessity of manganese for water oxidation [56] it is in all probability

a mangano protein. The experiments and results described in subsequent chapters pertain to PSII and therefore, structural considerations within the thylakoid membrane were limited to PSII. Information regarding PSI may be found in several reviews, for example [57].

B. Overview of Luminescence

Luminescence is primarily a PSII phenomenon. Because of nonradiative relaxation pathways, PSI fluorescence is not generally observed except at low temperatures [58]. Fluorescence studies have been one of the most extensively used techniques in the elucidation of PSII. Fluorescence changes following photochemistry have not only generated information pertaining to the chemical composition of PSII, but alterations of the behavior of fluorescence during redox titrations have provided accurate estimates for the midpoint potentials of various PSII electron carriers [59]. Upon illumination by a weak probe source, excitons are generated in the antenna system. The working definition of "weak" is such that illumination does not significantly affect the state of the sample. Upon generation, excitons may become "trapped" by P680 resulting in the photochemical oxidation of P680 and reduction of PQ_A. In addition, excitons may relax in either a radiative or nonradiative pathway with first order rate constants of k_f and k_{nr}, respectively. Simply stated, the fluorescence yield, Θ_{fo}, in dark adapted chloroplasts may be considered to be

$k_f/(k_f + k_{nr} + k_p)$, where k_p is the rate of photochemistry. Under conditions in which photochemistry is blocked, such as PQ_A being in the reduced state, the fluorescence yield increases and, assuming k_f and k_{nr} to be constant, the maximal fluorescence yield (Θ_{fm}) would be $k_f/(k_f + k_{nr})$. The difference between the maximal fluorescence intensity and that observed for dark adapted chloroplasts is referred to as the variable fluorescence (F_v) and is proportional to $\Theta_{fm} - \Theta_{fo}$. Thus, the fluorescence intensity should be most drastically affected by changes in the oxidation state of PQ_A; Θ_f is small when PQ_A is oxidized, large when PQ_A is reduced. The rate of change of the fluorescence yield is considered to follow the rate of change of the oxidation state of PQ_A [60]. Based on this simple model, the photochemical yield Θ_p ($k_p/[k_f + k_{nr} + k_p]$) would be:

$$\Theta_p = \frac{\Theta_{fm} - \Theta_{fo}}{\Theta_{fm}} = \frac{F_v}{F_m} \qquad \text{I-1}$$

where F_m is the maximal fluorescence intensity. Although this simple model is basically correct, it is incomplete and not valid under all experimental conditions. Realizing that the reaction center is distinct from the bulk chlorophyll, Butler refined this model (reviewed in [58]) to distinguish between the fate of an exciton when it is associated with the bulk chloroplyll from when it becomes "trapped" by the reaction center. The involvement of the reduction state of secondary acceptors of fluorescence

behavior, as well as the probability that the excitation energy may be transferred to another photosynthetic unit, renders an exact comparison of fluorescence kinetics to PQ_A difficult.

Several other factors are known to affect fluorescence. Nontheless, significant progress has been made in this area, particularly by Butler and coworkers [58]. As a result of the overlap of the absorption bands of the chlorophyll cation with the emission bands, $P680^+$ is a quencher of fluorescence [61], as is a carotenoid triplet which is formed following intense laser illumination [62].

In addition to absorption of light, excitons are generated by a process which may occur in the dark. The resulting afterglow or delayed luminescence (d.l.) has been studied from submicroseconds to several minutes following illumination. The spectral emission of d.l. is nearly identical to that observed for fluorescence with the maximum intensity centered around 685 nm. This suggests that d.l. and prompt fluorescence originate, in part, from the same pigment pool [63]. D.l. is further evidenced to be a PSII phenomenon by the enhanced intensity of d.l. observed for particles enriched in PSII and the absence of d.l. in PSI particles [64]. Although the addition of reductants results in a weak d.l. emission from dark adapted chloroplasts [65], preillumination of the sample is usually necessary in order to detect d.l. D.l. is generally believed to result from a reversal of normal photoinduced

electron transport [66]. Thus, based upon the recombination hypothesis the immediate precursor to d.l. should be the state [$P680^{+}PQ_A^{-}$]. Formally, the role of the pheophytin should be considered, but as pointed out by Klimov [67], the 5 ns phase of fluorescence rise may actually be due to a $P680^{+}PHEO^{-}$ back reaction. Thus for d.l. monitored at times greater than 100 ns following illumination, the role of the pheophytin species may be ignored.

The overall yield of d.l. is extremely small; estimates are on the order of 10^{-4} [68]. This low yield can be rationalized as follows: Photoabsorption generates the $P680^{+}$ PQ_A^{-} state. The forward rate of electron transfer, which generates stable charge separation, is large compared to the reverse recombination rate constant, k_b. Thus, the recombination yield is small; moreover, the generation of an exciton would occur in only a fraction (η) of the centers undergoing back reaction. Once generated, the exciton may relax radiatively with yield of Θ_1. Thus the instantaneous intensity of d.l., while extremely weak, is a measure of the concentration of $P680^{+}$ PQ_A^{-}. However to relate the kinetics of d.l. directly to the combined kinetics of $P680^{+}$ and PQ_A^{-} assumes that k_b, η, and Θ_1 are all constant. The work described in subsequent chapters will address these points. Emphasis will also be placed upon the effects of an external electric field on normal d.l. behavior.

CHAPTER 2

MATERIAL AND METHODS

A. Preparation of Samples

Class II chloroplasts were isolated from market spinach by the high-salt, low-salt method described by Robinson et al. [70]. This procedure consists of grinding a mixture of 500 to 1000 grams of washed, depetiolated spinach leaves and a high ionic strength solution (400 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 20 mM HEPES, pH 7.5) for 10 seconds in a Waring blender. To remove membrane and leaf fragments, the homogenate was filtered through 10 layers of cheesecloth and centrifuged at 1000 × g for 20 s. The supernatant was then centrifuged at 4000 × g for 10 minutes; the resultant pellet consisted of isolated thylakoids. The thylakoids were then subjected to a low salt wash by suspending them in about 50 ml of a 20 mM HEPES (pH 7.5), 150 mM NaCl, 4 mM $MgCl_2$ solution followed by a 10 minute centrifugation at 4000 × g. After the low salt wash, the class II chloroplasts were suspended in SHN (400 mM sucrose, 10 mM NaCl, 50 mM HEPES, pH 7.5) to a final volume of 25 to 50 ml such that the chlorophyll concentration was about 3 mg/ml. Integrity of the photosynthetic apparatus was determined by measuring the rate of oxygen evolution with typical rates

being between 200 and 250 μmole O_2/mg_{Chl}-hr. Following isolation, the chloroplasts were frozen at -40°C, and stored until needed. Freezing of the sample did not significantly affect the activity of the chloroplasts as indicated by the rates of steady state oxygen evolution in Table 1. and the relative O_2 flash yields plotted in Figure II-1. In order to minimize any long term degradation effects, chloroplasts were usually used within two weeks following isolation. During the isolation procedure, care was taken to keep the sample cold (4°C).

Table 1

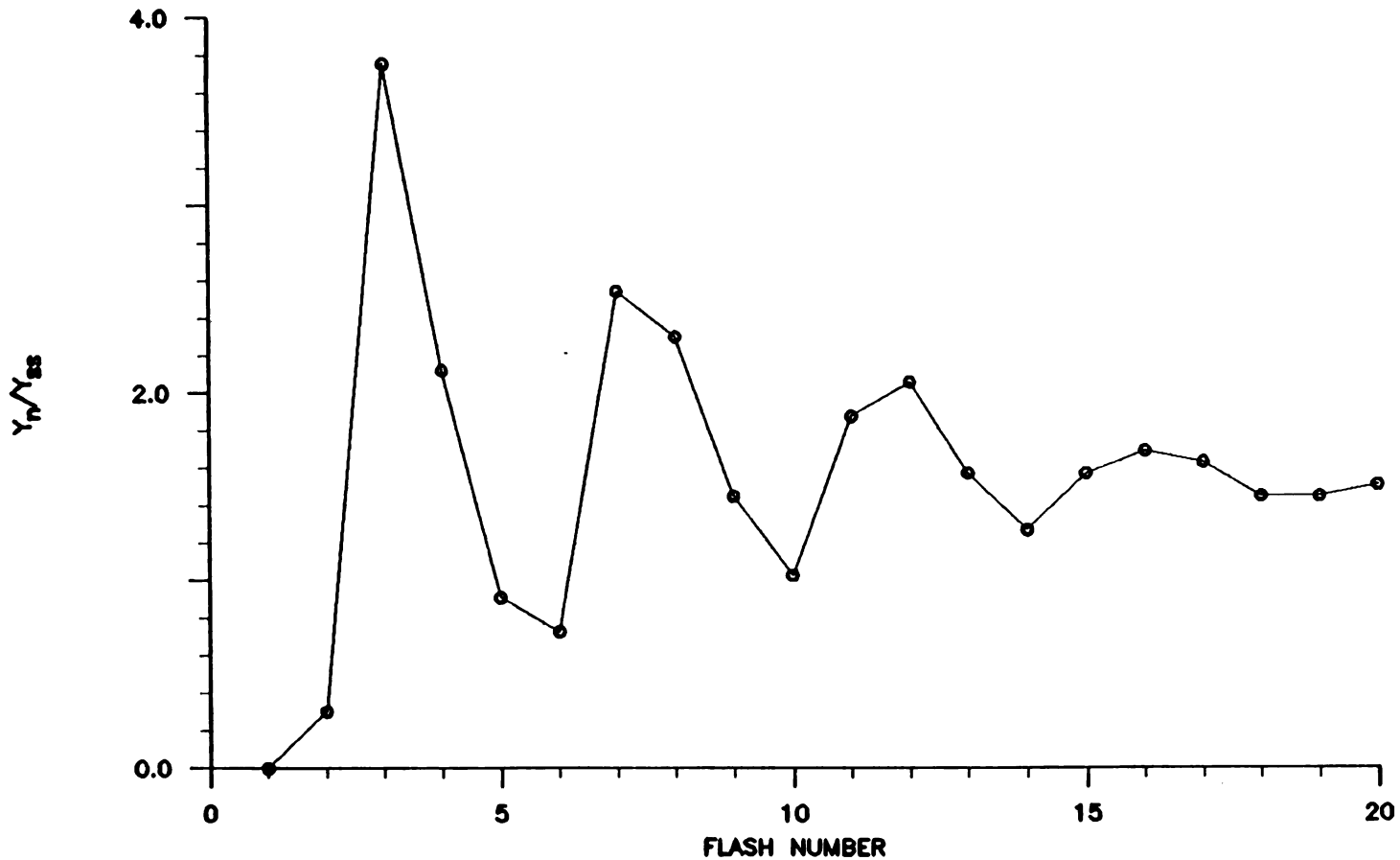
treatment	rate $\frac{\mu mole\ O_2}{mg_{Chl}\ hr}$
freshly isolated	255
frozen at -40°C for 40 days	240

A method to isolate Photosystem II fragments capable of evolving oxygen at a rate between 270 to 350 μmole O_2/mg_{Chl}-hr while being essentially void of Photosystem I activity was recently developed [71]. Following the low-salt wash, class II chloroplasts were incubated in the dark and on ice for 30 minutes in a solution of 50 m$\underline{M}$ HEPES (pH 7.5), 15 m$\underline{M}$ NaCl, 5 m$\underline{M}$ $MgCl_2$, 1 m$\underline{M}$ ascorbate, 2 mg/ml BSA and 50 mg/ml Triton X-100 detergent at a chlorophyll concentration of 2 mg/ml. Following the Triton incubation the PSII fragments were pelleted by a 30 minute centrifugation at 40,000 × g. The

FIGURE II-1

Flash oscillations in oxygen yield observed for chloroplasts which were stored at -40°C. The light source was a flash-lamp (Stroboslav) which provides 20 μs light pulses. The sample was illuminated at a repetition rate of 1 flash per second. The reaction media consisted of 0.4 M sucrose, 50 mM HEPES, and 10 mM NaCl. The sample contained approximately 1.5 mg/ml chlorophyll.

RELATIVE O_2 YIELD vs. FLASH NUMBER



pellet was suspended in SHN plus 10 mg/ml Triton X-100 at a chlorophyll concentration of 2 mg/ml. This suspension was centrifuged at 40,000 × g for 30 minutes. The pellet was then suspended in SHN plus 10 m$\underline{M}$ $MgCl_2$ and stored at -40°C. The chlorophyll concentration in this suspension was adjusted to be around 3 mg/ml.

Tris-EDTA washed chloroplasts, which have electron transport between the OEC and Z inhibited, were prepared by suspending isolated thylakoids obtained after the low-salt wash in a solution of 0.8 $\underline{M}$ tris (pH 8.0) and 1 m$\underline{M}$ EDTA. Following a 20 minute incubation period at 4°C in the presence of normal room light, the chloroplasts were pelleted by a 10 minute, 4000 × g centrifugation. The tris-treated chloroplasts were washed by suspending in SHN followed by a second 10 minute, 4000 × g centrifugation. The pellet was suspended in SHN at a chlorophyll concentration of 3 mg/ml.

In each preparation, the chloroplyll assay was done according to the method of Sun and Sauer [72]. An aliquot of the chloroplast stock solution was diluted 200-fold in 80%/20% acetone/water. After filtering insoluble components (Whatman number 1 filter paper), the absorption at 652 nm was measured by using either a McPherson Spectrometer or a Beckman DU spectrometer and a 1 cm cuvette; the absorbance multiplied by 5.8 yielded the chlorophyll concentration of the stock solution in mg/ml. This procedure produced

identical results for total chlorophyll content to those obtained from the method of Arnon [73].

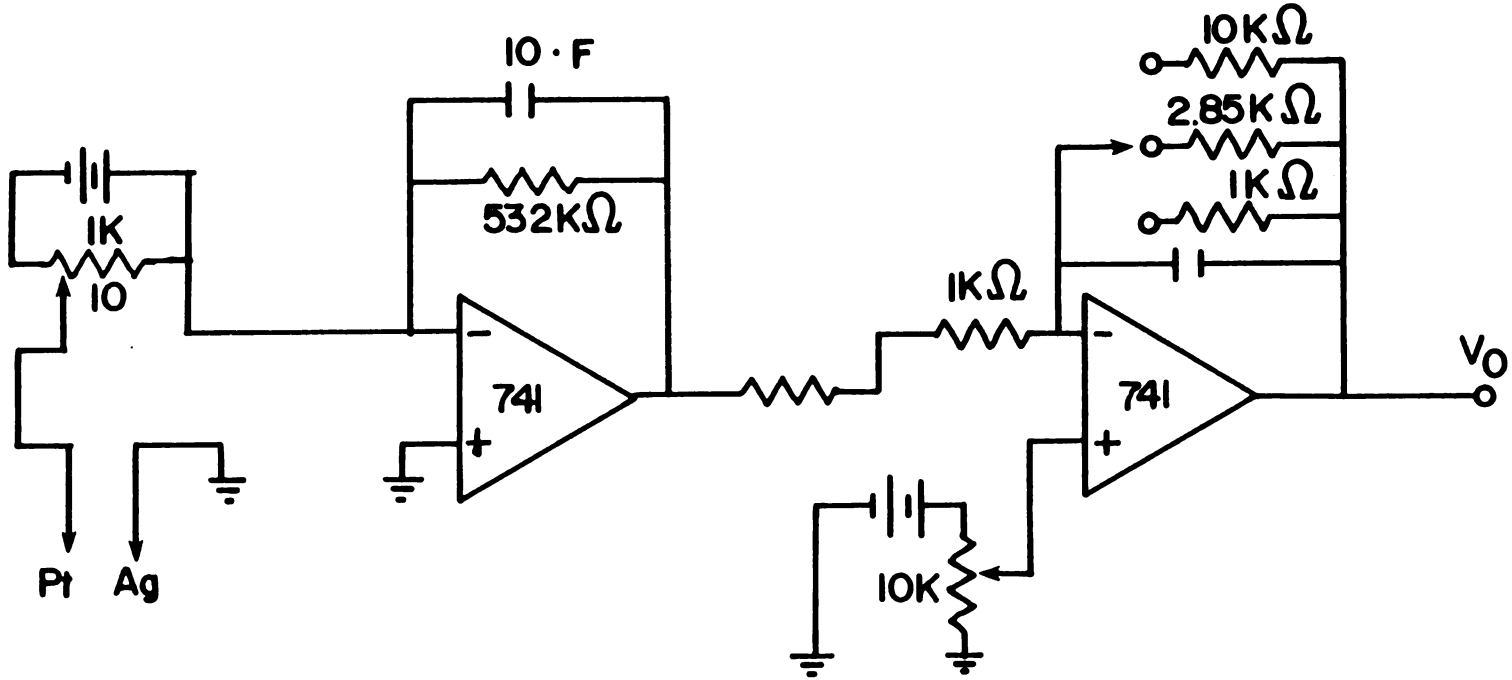
The rate of oxygen evolution was measured polarographically by using a YSI 5331 Oxygen Probe and initially the circuit shown in Figure II-2 interfaced to a strip chart recorder. The detection circuitry was subsequently replaced by a YSI model 53 monitor. The light source was a GE EJL lamp used in conjunction with a heat filter. For chloroplasts, the reaction medium consisted of 20 m$\underline{M}$ HEPES (pH 7.5), 50 m$\underline{M}$ NaCl, 2 m$\underline{M}$ $MgCl_2$, with 2.5 m$\underline{M}$ $K_3Fe(CN)_6$ as an electron acceptor and 10 m$\underline{M}$ methylamine as an uncoupler; for the PSII particles, the reaction medium was 20 m$\underline{M}$ MES (pH 6.0), 50 m$\underline{M}$ NaCl, 2 m$\underline{M}$ $MgCl_2$, and 3.5 m$\underline{M}$ $K_3Fe(CN)_6$ with 0.285 m$\underline{M}$ 2,5-dichloro-p-benzoquinone as acceptors. For each assay, the chlorophyll concentration was typically 10 to 30 μg/ml. Flash number oxygen yields were measured on an electrode similar to the design of Joliot [74].

B. Apparatus

A block diagram of the apparatus used to measure delayed luminescence and the luminescence induced by an externally applied electric field (EPL) is shown in Figure II-3. The actinic light source used in most of the experiments described in subsequent chapters was the second harmonic of a Q-switched Nd/YAG (Quanta-Ray model DCR) laser, which produces radiation at 530 nm with a pulse width of 20 ns. Other actinic sources which were used included

FIGURE II-2

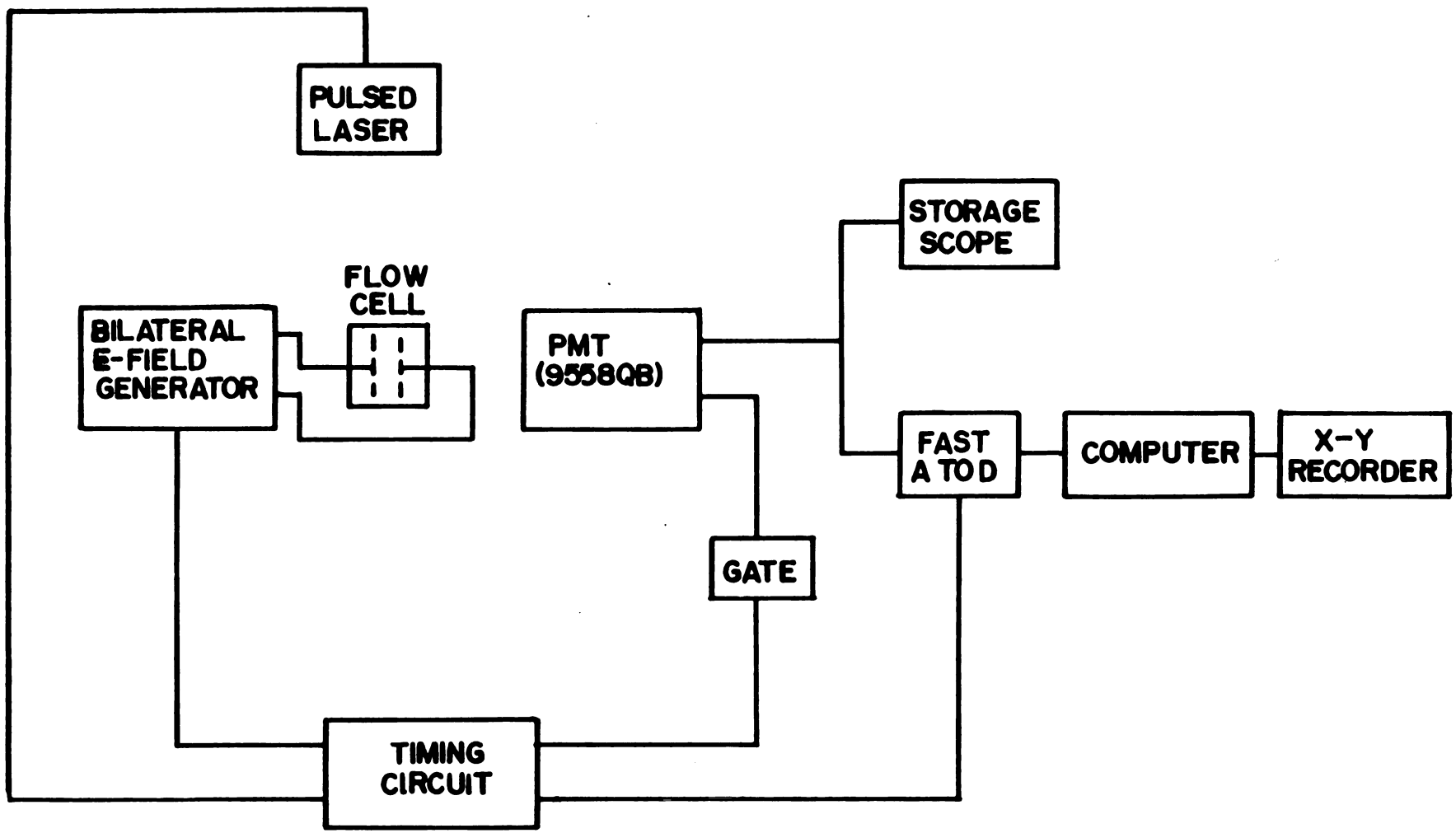
Polarographic circuit used to monitor steady state oxygen evolution. Platinum (Pt) and Ag/AgCl (Ag) electrodes were used to detect oxygen evolution from illuminated chloroplasts.



O_2 ELECTRODE

FIGURE II-3

Block diagram of the EPL/d.l. generation and detection apparatus. Details given in the text.


PULSED LASER
STORAGE SCOPE
FLOW CELL
BILATERAL E-FIELD GENERATOR
PMT (9558QB)
FAST A TO D
COMPUTER
X-Y RECORDER
GATE
TIMING CIRCUIT

xenon flash lamps (Stroboslave) and a 450 ns pulsed dye laser (Phase R model dl 1100). The xenon lamps were not intense enough to saturate photochemistry, whereas the dye laser was plagued by poor shot-to-shot reproducibility and rapid degradation of the dye (rhodamine 590). These were not significant problems with the Nd/YAG laser. For each source, the actinic light was passed through an array of optical filters that included an IR heat absorbing filter and a Corning CS4-96 filter. In addition, a 600 nm short pass filter (Baird-Atomic) was used in conjunction with the xenon lamp and dye laser. For light saturation studies, the actinic light intensity was attenuated using calibrated neutral density filters (Baird-Atomic). Also included in the actinic optics were a pair of lenses which served to telescope the beam diameter to the size of the window of the sample flow cell. Illumination of the sample was through the top of the cell; this was facilitated through the use of a beam raiser. A schematic of the optical arrangement used is depicted in Figure II-4.

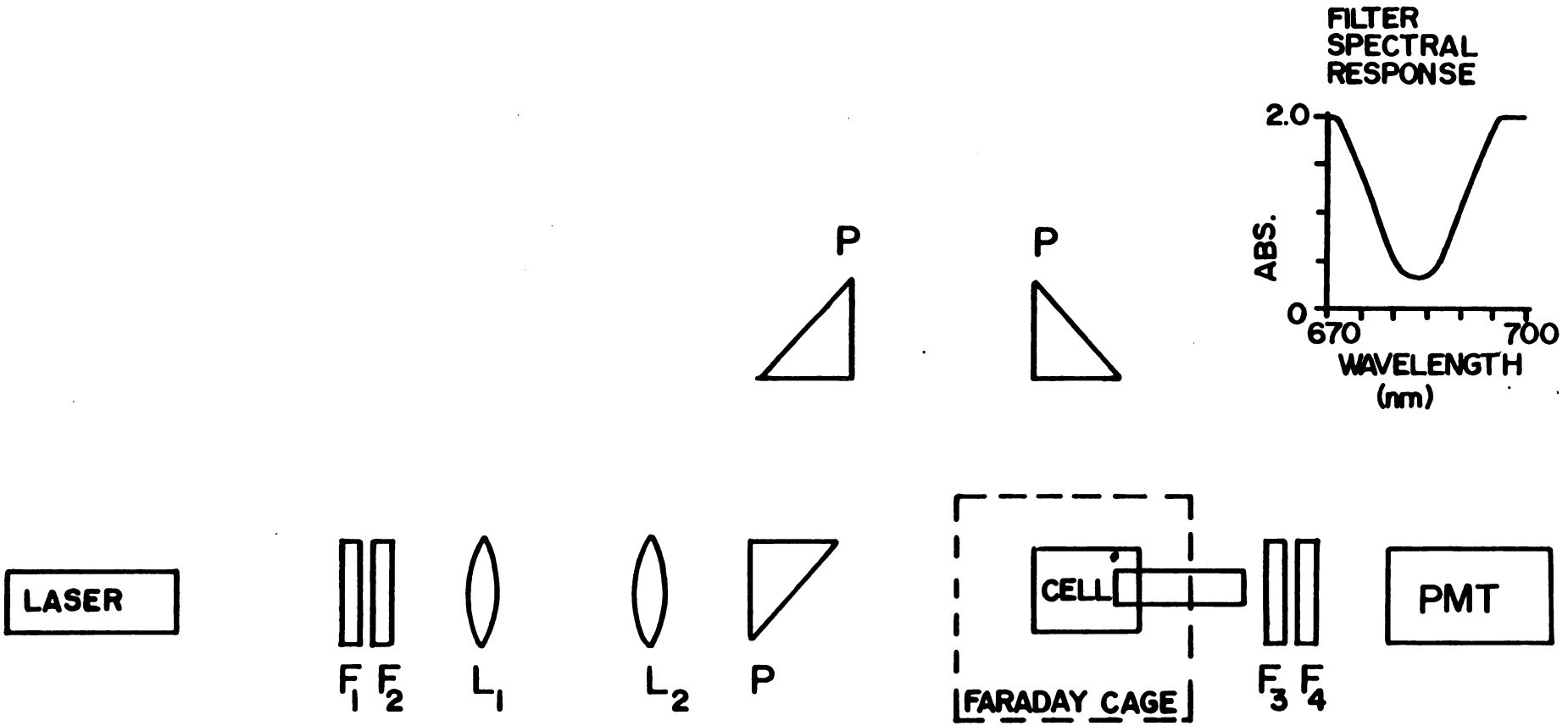
The sample cell, which was sequestered in a Faraday cage to minimize interference from electrical transients and ambient light, was constructed of black plexiglass except for two mutually perpendicular windows which allow for monitoring of the emanating luminescence 90° to the actinic light. Additionally, perpendicular to the polished plexiglass windows of the cell, were two parallel electrodes, which were separated by distances of 0.25, 0.50, or 1.00 cm

depending upon the particular cell design. It is across these electrodes that the bilateral Electric Field Generator produces the electric field. This was achieved by holding each electrode at a common potential (adjustable between 0 to 900 V). A similar cell design was initially used for temperature jump experiments [75]. At a predetermined time relative to laser illumination, the potential of one electrode is dropped to ground, thus generating a potential drop across the electrodes. Associated with this potential drop is the external electric field, the magnitude of which is the potential drop divided by the thickness of the cell. The duration of the field may be adjusted from 10 to 10,000 μs; the rise and decay time of the field (from 10 to 90% on) was measured to be 1 μs. A second field may be applied on the sample with variable delay times between 10 μs and 1 s. The polarity of the second field can be in either the same or opposite direction as the first. The delayed luminescence and EPL was directed toward the photomultiplier (PMT) with an extended S-20 response (EMI-9558 QB) via a polished lucite light pipe. Sandwiched between the light pipe and the photocathode surface of the PMT was a combination of a 685 nm bandpass filter (Baird-Atomic) and a Schott RG-665 red cutoff filter. The spectral response of the protection filters is shown in Figure II-4.

To avoid saturation of the PMT and its detection circuitry during the intense laser illumination, a photomultiplier gate similar to the design of Groves [76]

FIGURE II-4

Optical arrangement of the EPL/d.l. apparatus. F1, heat absorbing filter; F2, corning CS 4-96 filter; F3, 685 bandpass filter; F4, Schott RG665 filter. More details given in the text. The insert displays the spectral response of the PMT protection filters (F3 and F4).



OPTICAL ARRANGEMENT
FOR D.L. APPARATUS

was used. The PMT could be gated off for variable times (3 to 70 μs) as determined by the duration of the duty cycle of a +5 V square wave. The PMT amplifier was designed for fast time response (<1 μs) and high gain. The output of the PMT was digitized and stored in 1024 successive channels by using either an 8-bit analog to digital (A to D) converter with a channel advance of 1, 2, 5, or 10 μs, or a 7-bit A to D with a channel advance of 60, 100, 200, or 500 ns. The digitizer was interfaced to a signal averager (Nicolet model 1072) where the results of several experiments were summed. The purpose of the auxilary A to D converter was to improve the time response of the system. The fastest sampling time of the Nicolet 1072 was 20 μs per channel, whereas the fast A to D could sample at a rate of up to 15 MHz. The final results of an experiment were printed on an X-Y recorder (Hewlett Packard model 7001). Proper sequential triggering of each of the components of this apparatus was achieved by utilizing a timing circuit that ran on a 1 MHz clock.

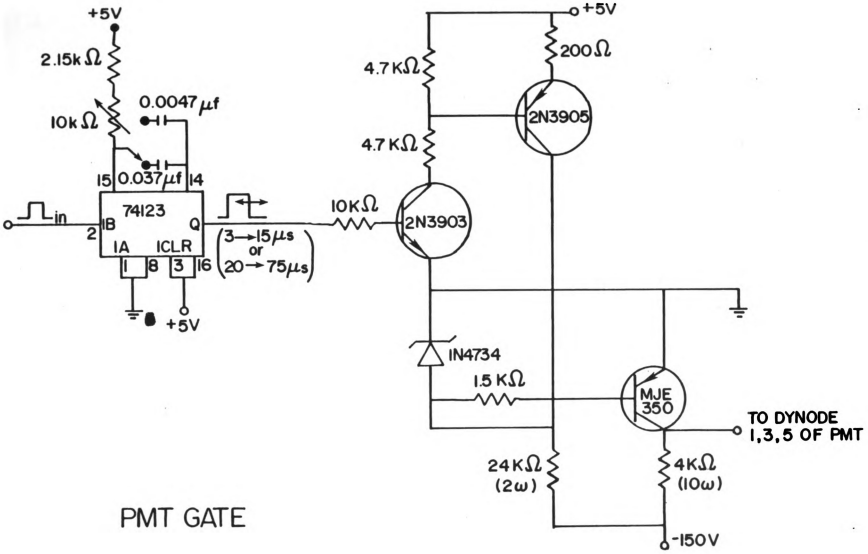
The timing circuit, electric field generator, fast A to D, and the PMT gate and amplifier were of in-house design. Schematics of the PMT amp and gate are shown in Figures II-5 and II-6.

C. Experimental Protocol

Once thawed, the stock solutions of the isolated thylakoids were stored on ice and in the dark. Aliquots

FIGURE II-5

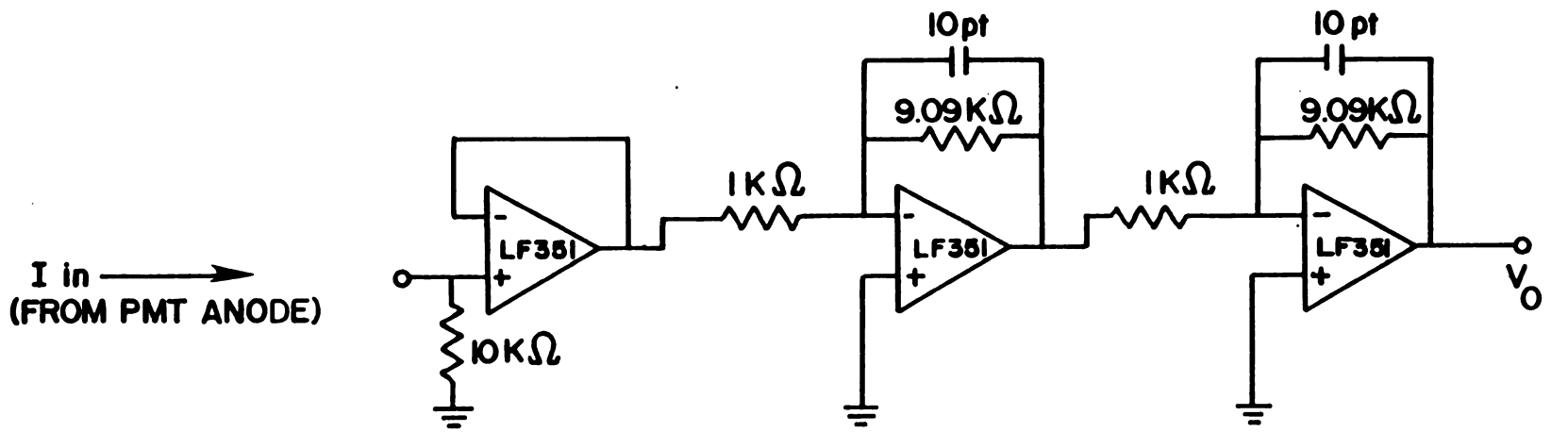
PMT gating circuit. The circuit is a modification of the gating circuit described by Groves [76].


+5V
2.15kΩ
10kΩ
0.0047μf
0.037μf
15
14
74123
2
1B
Q
1A
ICLR
1
8
3
16
+5V
in
(3→15μs or 20→75μs)
10KΩ
2N3903
4.7KΩ
4.7KΩ
+5V
200Ω
2N3905
IN4734
1.5KΩ
MJE 350
TO DYNODE 1,3,5 OF PMT
24KΩ (2ω)
4KΩ (10ω)
-150V

PMT GATE

FIGURE II-6

PMT amplifier circuit.



PMT AMPLIFIER

of this stock solution were diluted approximately 750-fold to a chlorophyll concentration of 5 μg/ml in a reaction medium of 10 mM buffer. The various buffers used were succinate (pH 4.5), MES (pH 5.2, 6.0), HEPES (pH 7.0, 8.0), and tricine (pH 9.0). Following a 5 minute dark incubation period, the delayed luminescence or EPL induced by a sequence of light pulses was measured. Typically, each d.l. experiment was the average of 10 measurements. Because of the enhanced signal magnitude, EPL experiments were typically the average of 5 measurements. All experiments were performed at room temperature. Following the measurement, the sample within the cell was purged by using a peristalic pump to flow fresh sample into the cell. The duration of time during which the pump was on was controlled by the delay time between two input pulses in the circuit shown in Figure II-7. In order to minimize heating and electrophoresis effects in an EPL experiment, the sample was never subjected to more than one electric field pulse. In a typical experiment, the field was applied for 50 μs. Generally, there was a small background signal due to scattering of light off the membrane vesicles and an inefficiency of the filters used. Correction for this artifactual background signal was done by measuring a blank signal obtained from a heated (5 minutes at 70°C) sample and subtracting this blank signal for the signal obtained from active samples. Figure II-8 shows the relative intensity of d.l. compared to the artifactual signal.

FIGURE II-7

Circuit designed to control the duration of time during which the peristalic pump was on. This time was set by the delay time between the two TTL input pulses.

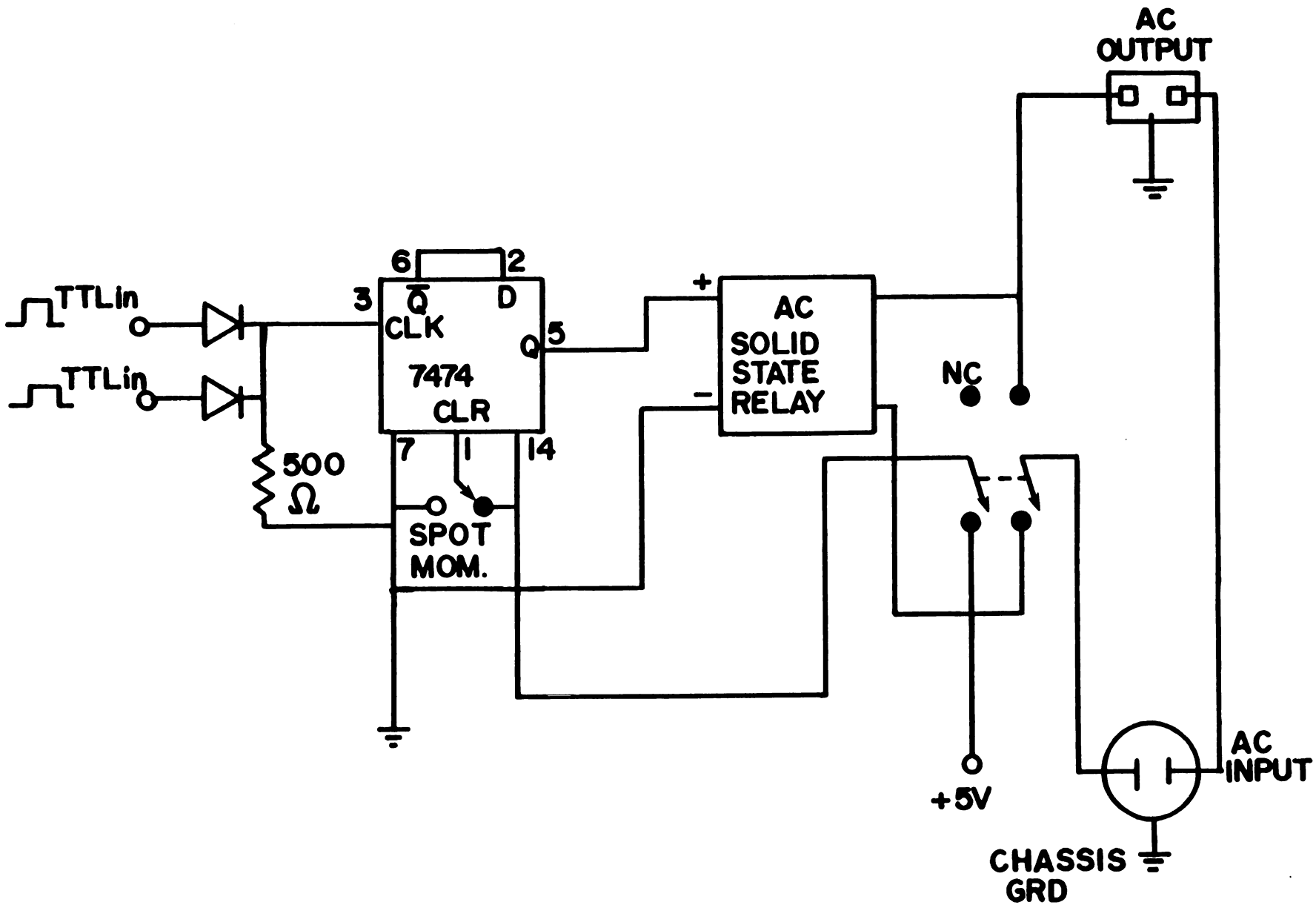

AC
OUTPUT
TTLin
TTLin
6
2
3
Q
D
CLK
5
Q
7474
CLR
7
1
14
500
Ω
SPOT
MOM.
+
−
AC
SOLID
STATE
RELAY
NC
+5V
AC
INPUT
CHASSIS
GRD

FIGURE II-8

A comparison of the d.l. signal (B) obtained from dark adapted, tris-washed chloroplasts at pH 7.0 to the artifactual signal illuminated by a single saturating laser flash. Aside from the use of heat treated chloroplasts (5 minutes at 70°C) for trace A, trace A and trace B were measured under identical conditions. Each trace is the average of 10 measurements.

D. Analysis of the Data

By utilizing an electronic digitizer on the kinetic traces obtained for the delayed luminescence measurements, point values for the intensity of d.l. as a function of time were obtained. The typical sampling rate corresponded to one point per μs. These data were analyzed by using a nonlinear regression analysis routine [77] on file in the Michigan State University Computer Library. Typically, the d.l. analysis was in terms of a biphasic decay. The results of these analyses are described in Chapter 3. The effect of an external electric field was usually analyzed in terms of the maximal enhancement of luminescence which resulted from the external field relative to the level of d.l. in the absence of the perturbation. The magnitude of this enhancement is referred to as maximal EPL intensity.

CHAPTER 3

DELAYED LUMINESCENCE

A. Overview of Microsecond Delayed Luminescence

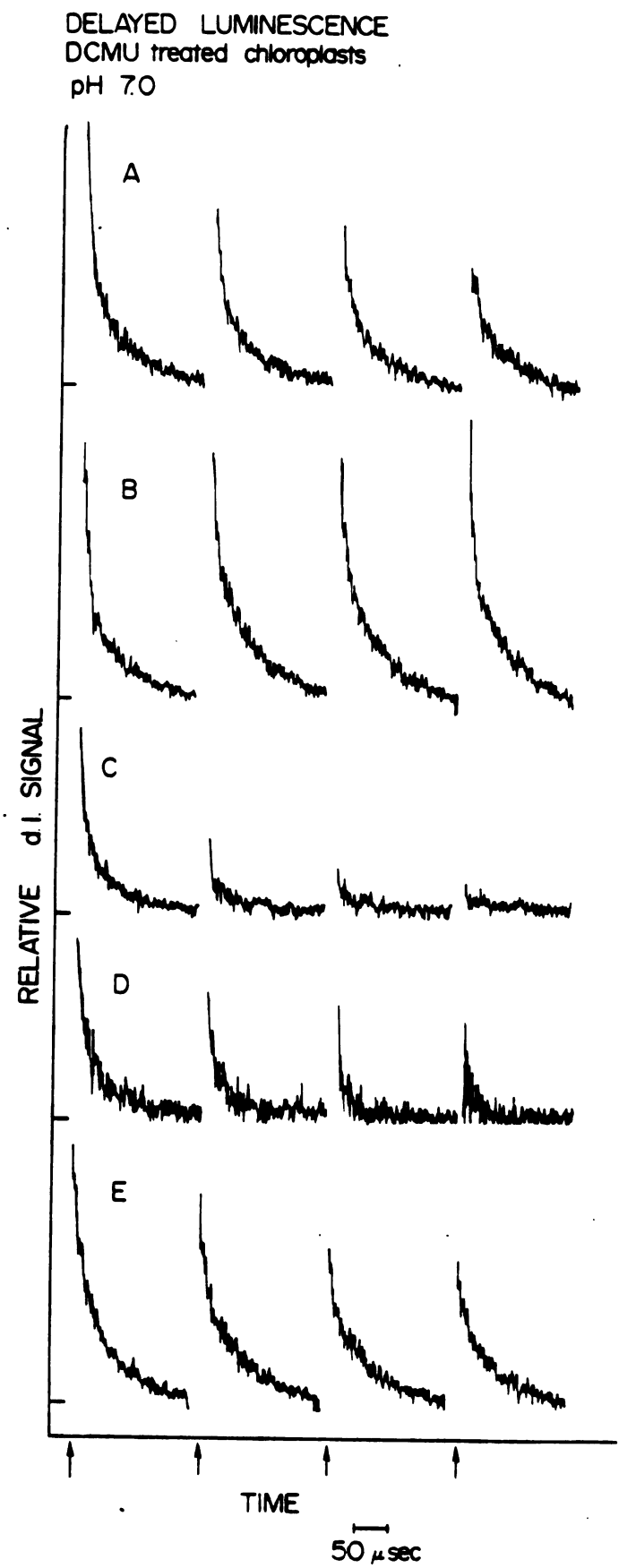
Delayed luminescence (d.l.) is ascribed to the reversal of the electron transfer reactions of Photosystem II. It is, however, generally accepted that the recombination hypothesis has limitations. Prior to investigating the effect of an external electric field on PSII as monitored by an induced luminescence, it became apparent that it was necessary to quantify these limitations. In order to ascertain the extent to which nonperturbed d.l. is a direct indicator of PSII events, d.l. measurements were performed under a variety of conditions. The results of these experiments are presented in this chapter.

As described in the introduction, delayed luminescence in green plants requires functionally active Photosystem II centers; treatments which inhibit PSII photochemistry tend to quench d.l. This quenching effect is demonstrated in Figure III-1a which shows d.l. from tris-EDTA washed, DCMU treated chloroplasts following a series of short actinic flashes. In these samples, electron transport between the OEC and Z and between PQ_A and PQ_B is inhibited.

FIGURE III-1

D.1. from dark adapted DCMU treated chloroplasts (5 μg/ml) in 10 m$\underline{M}$ HEPES (pH 7.0) following each of 4 actinic flashes (1 flash/second). Following the 4th flash, a fresh sample was flowed into the cell. Each trace is the average of 10 experiments.

A) Tris-EDTA washed, DCMU treated chloroplasts. No additions. Approximate gain = 1.
B) Tris-EDTA washed, DCMU treated chloroplasts plus 3 m$\underline{M}$ ferricyanide and 10 m$\underline{M}$ Mg^{2+}. Approximate gain = 1.
C) Tris-EDTA washed, DCMU treated chloroplasts plus 0.02 m$\underline{M}$ hydroquinone/1 m$\underline{M}$ ascorbate. Approximate gain = 1.
D) DCMU treated chloroplasts. No additions. Approximate gain = 2.0.
E) DCMU treated chloroplasts plus 3 m$\underline{M}$ ferricyanide and 10 m$\underline{M}$ Mg^{2+}. Approximate gain = 1.


DELAYED LUMINESCENCE
DCMU treated chloroplasts
pH 7.0
A
B
C
D
E
RELATIVE d.l. SIGNAL
TIME
50 μsec

Schematically, PSII may be represented as:

$$ZPQ \underset{k_b}{\overset{h\nu}{\rightleftharpoons}} ZP^+Q^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Z^+PQ^- \qquad \text{III-1}$$

where for brevity PQ_A is abbreviated further to Q, P represents the P680-Pheophytin complex, and k_1, k_{-1} and k_b are the rate constants for the reduction of P^+ by Z, the reduction of Z^+ by P and the P^+Q^- backreaction, respectively. ZPQ represents the dark adapted state, ZP^+Q^- is formed in 200 ps following laser illumination [15]. P^+Q^- is stabilized by the rapid reduction of P^+ by Z ($t_{½} << 100$ μs). In dark adapted samples the majority of the centers are open, that is, PQ_A is in its oxidized form. There is, however, evidence that about 33% of the centers are closed (PQ_A reduced) as a result of a DCMU mediated reduction of PQ_A by the fraction of PQ_B in the semiquinone form [78]. As the system undergoes photoinduced turnovers, the d.l. is quenched. This quenching arises from an incomplete relaxation of the Z^+PQ^- state to the ZPQ state as well as the reduction of Z^+ to produce the ZPQ^- state. Those centers in which PQ_A is reduced are incapable of undergoing photochemistry, and hence the generation of d.l. is inhibited. Electron donation to Z^+ may occur via endogenous donors which undergo oxidation only under conditions in which normal electron transport to the OEC is blocked. Spinach chloroplasts, for example, contain significant amounts of ascorbate which can serve as a System II donor when the OEC is inhibited. Reduction

of Z^+ by these endogenous donors could be controlled primarily by kinetic factors since the lifetime of the oxidized form of Z increases by over 4 orders of magnitude in tris-washed chloroplasts relative to intact oxygen evolving samples [79]. The addition of exogenous electron acceptors such as ferricyanide coupled with Mg^{2+} to shield the membrane surface charge essentially reverses the quenching effect of the multiple turnovers (Fig. III-1b). Mg^{2+} alone did not alter the behavior of d.1. In contrast to electron acceptors, the addition of the electron donor hydroquinone (0.2 m$\underline{M}$ plus 1.0 m$\underline{M}$ ascorbate) significantly quenches the d.1. by the fourth flash (Fig. III-1c). Similar effects may be observed on DCMU treated samples with electron transport from the OEC still intact (Figs. III-1d and III-1e). In the absence of acceptors, the intensity of d.1. decreases greatly with flash number; this quenching is a reflection of the efficiency of Z^+ reduction by the OEC. This effect is only partially reversed by the addition of ferricyanide coupled with 10 m$\underline{M}$ Mg^{2+}.

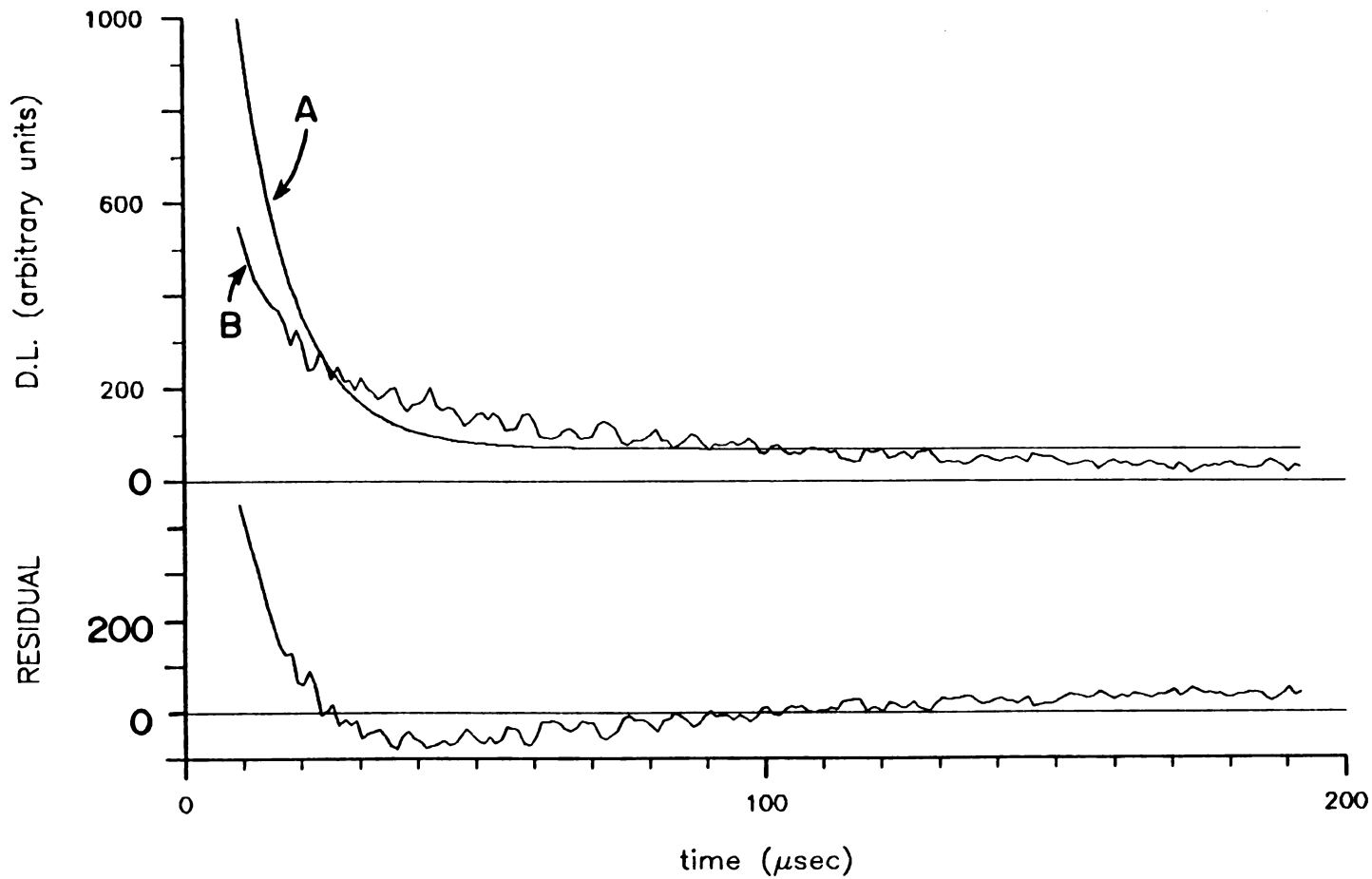
D.1. thus appears to arise from a reversal of the photoinduced electron transport reactions of PSII. However attempts to simulate the kinetic behavior of d.1. mathematically from the simple electron transfer model described by Eq. III-1 by using accepted values for k_1 and k_b to experimental data did not give satisfactory results. This is shown in Fig. III-2 in which the experimental results are compared to the theoretical curve. The systematic

FIGURE III-2

A comparison of the experimental d.l. decay in tris-EDTA washed, DCMU treated chloroplasts (B) induced by a single saturating laser flash to that predicted by equation III-1 (A). The lower curve is a plot of the residual (A-B). Chapter 5 describes the model (curve B) in detail. The specific parameters for the simulation were: k_1 and k_b corresponded to halflives of 7 and 120 μs (from references 23 and 69, respectively). k_{-1} was an adjustable parameter and had a value corresponding to a halflife of 250 μs.

DELAYED LUMINESCENCE

Tris–EDTA washed, DCMU treated chloroplasts
pH 7.0, Flash 1



residual plot indicates the inadequacies of a simple electron transport model to describe delayed luminescence. It should be noted, however, that there are extensive reports in the literature [31] on the kinetic behavior of $P680^+$ in dark adapted tris-washed chloroplasts. The effect of DCMU treatment in tris-washed chloroplasts on rates of electron transport has not been as thoroughly investigated. Thus the rate constants used in the simulation were obtained from the reports on tris-washed chloroplasts. Although DCMU inhibition should not significantly affect $P680^+$ kinetics, some of the discrepanices of Figure II-2 may arise from its presence. The model is described in detail in Chapter 5.

D.l. is, however, sensitive to the state of PSII. Zankel [80] was the first to demonstrate that the intensity of d.l. observed in the submillisecond time range following flash illumination followed a damped oscillatory behavior similar to that observed for oxygen evolution with the maximum intensity on the third flash. This is in contrast to millisecond d.l. which displayed maximum intensity on the second flash [81]. In *Chlorella*, the relative intensity and kinetics of μs d.l. following flashes 2 and 3 were such that at times greater than 5 ms, the yield induced by the second flash was greater than the third [81]. This is apparently due to the kinetics associated with the reduction of Z^+ by the S_3 state of the OEC to form S_4; generation of S_4 results in the irreversible oxidation of water.

In attempts to correlate the effect of pH on PSII electron transport, Bowes and Crofts [82] investigated the flash number dependence of d.l. (from 7 to 200 μs) in the pH range 4.0 to 9.0. They reported that in the pH range of 6.0 to 8.0, the d.l. intensity decayed biphasically with pH independent halftimes of 10 to 15 and 40 to 50 μs. The total initial intensity and the amplitude of the fast phase displayed a distinct 4 flash oscillatory behavior. Oscillations in the amplitude of the slower phase were not as apparent. At pH 4.0, the d.l. intensity was independent of flash number and decayed with a halftime of 135 μs. In contrast, at pH 9.0, the d.l. intensity on the first flash was quenched; the intensity increased with flash number until leveling off at a maximum value following the fifth turnover. The effect of tris-washing on μs d.l. relative to that observed in untreated chloroplasts was reported by Jursinic and Govindjee [83]. The experimental protocol differed from that of Bowes and Crofts [82] in that d.l. was monitored by a train of actinic light pulses given at various repetition rates. At a flash repetition rate of 1 flash per 5 seconds, the d.l. from tris-washed samples was nearly identical to that observed from untreated chloroplasts. In tris-washed chloroplasts, the decay time of the fast phase was independent of the rate of illumination with a reported halftime of 6 μs. The life-time of the slower phase of d.l.. however. decreased as the dark time between flashes increased. The results of

Bowes and Crofts [82] and Jursinic and Govindjee [83] were analyzed in light of reports by Den Haan et al. [35] in which $P680^+$ reduction was reported to decay with a submicrosecond halftime and by Blankenship et al. [37] in which the rise time of Signal IIvf was reported to be 15 μs. Hence,to account for the biphasic behavior of d.l. the existence of 2 intermediate electron carriers between the OEC and P680 was postulated:

$$\text{OEC} \longrightarrow \text{Z1} \longrightarrow \text{Z2} \longrightarrow \text{P680} \qquad \text{III-2}$$

In this model,the oxidation of Z1 gives rise to Signal IIvf in active chloroplasts or to Signal IIf in samples with inhibited oxygen evolution. To account for the low intensity of d.l. in dark adapted samples at pH 9.0, it was postulated that at high pH electron transport is blocked between Z1 and Z2 and that there existed an electron donor to P680 which acted in parallel to Z2. This donor did not supply the OEC with oxidizing equivalents. However, the recent ESR experiments described in the introduction in which the decay of $P680^+$ complements the rise of Z^+ in tris-inhibited PSII fragments [40], and the optical studies reported by Conjeaud and Mathis [31] indicate that there is only a 1 electron capacity on the oxidizing side of PSII in tris-washed chloroplasts. These results, coupled with the possibility that the risetime of Signal IIvf reported in [37] may have been obscured by an ESR signal originating from PSI, indicate that Z1, the Signal II

precursor, reduces $P680^+$ directly. The μs kinetics of d.l. will be reexamined in view of these findings.

B. Results

1. D.l. from Dark Adapted Tris-Washed Chloroplasts

In order to correlate PSII phenomenon to d.l., it proved to be more convenient to use tris-EDTA washed chloroplasts which were not DCMU poisoned. Although DCMU treatments which block secondary electron transport on the reducing side of PSII should simplify kinetic behavior, the effect of tris treatment without additional inhibitors is better characterized in the literature.

According to the recombination hypothesis, the immediate precursor of d.l. may be considered to be the state $P680^+PQ_A^-$. The kinetics of $P680^+$ in tris-washed chloroplasts were extensively studied by Mathis and coworkers [25,84]. In dark-adapted, tris-treated samples, the kinetics of the submillisecond reduction of $P680^+$ were analyzed in terms of a biphasic decay. The results indicated the presence of a dominant fast pH dependent phase and a minor slow pH independent phase. The fast phase varied from a reported halflife of 3.5 μs at pH 8.0 to 44 μs at pH 4.0. The halflife of the slow phase was 100 to 200 μs throughout the pH range studied. At pH 9.0, the $P680^+$ halflife was less than 2 μs, and because of limitations in the instrument response time it could not be resolved. Similar pH dependent kinetics are observed for

d.l. (in the time range of 7 to 200 μs) following a single actinic flash in tris-treated samples. Table 2 summarizes the d.l. data obtained between pH 4.5 and 8.0 in dark adapted samples. Normalized experimental traces are shown in Figure III-3. Owing to chloroplasts clumping at low pH, no d.l. experiments were performed below pH 4.5. D.l. measurements at pH 9.0 were irreproducible, presumably because of the rapid reduction of $P680^+$ ($t_{\frac{1}{2}} < 2$ μs) coupled to the approximately 7 μs recovery time that was necessary for the detection apparatus following PMT gating and laser illumination. In Bowes and Crofts [82], the low d.l. intensity at pH 9.0 following a single flash was ascribed to a parallel donor to Z which becomes dominant as a result of a high pH induced inhibition of electron transport between the OEC and P680. Since chloroplasts suspended at pH 9.0 were still able to generate Z^+ as monitored by ESR Signal IIf, the parallel donor model is probably incorrect. Thus, the fast phase of d.l. under these conditions appears to be a measure of reactions associated with $P680^+$ reduction, the kinetics of PQA^- oxidation is over an order of magnitude slower. That PQA^- does not significantly affect the fast phase of d.l. decay is further substantiated by the identical pH dependent behavior of the fast phase of d.l. observed for tris-EDTA, DCMU inhibited chloroplasts as shown in Figure III-4.

A significant difference between the kinetics of d.l. and $P680^+$ decay is the absence of a pH independent 100-200 μs

TABLE 2 Delayed Luminescence Kinetic Behavior Induced by a Single Saturating Laser Flash.[a]

pH	A_{ext} [b]	A_{10} [c]	$t_{½}$(fast) [d]	$t_{½}$(slow) [e]	Fraction (fast) [f]
			UNTREATED		
4.5	1975 ± 772	1642 ± 600	18.3 ± 1.36	89.0 ± 7.5	0.37 ± 0.03
5.2	1606 ± 319	1254 ± 215	17.0 ± 4.9	73.2 ± 7.0	0.53 ± 0.07
6.0	817 ± 258	514 ± 118	8.9 ± 1.7	48.8 ± 1.0	0.56 ± 0.02
7.0	1298 ± 221	673	7.1 ± 1.7	38.6 ± 4.7	0.65 ± 0.08
8.0	1181 ± 240	657 ± 95	4.9 ± 0.8	52.0 ± 22.0	0.77 ± 0.07
			TRIS-EDTA WASHED		
4.5	1467 ± 525	1167 ± 300	17.9 ± 0.6	79.0 ± 0.82	0.49 ± 0.08
5.2	1604 ± 276	1239 ± 137	14.9 ± 1.6	76.0 ± 12.1	0.58 ± 0.08
6.0	1247 ± 203	902 ± 137	12.6 ± 1.92	66.0 ± 12.9	0.61 ± 0.08
7.0	1793 ± 150	1000	7.6 ± 1.1	45.8 ± 3.9	0.64 ± 0.04
8.0	1678 ± 624	735 ± 107	5.8 ± 1.3	41.6 ± 8.7	0.73 ± 0.05

[a] The uncertainties represent standard deviations. The values listed in the columns A_{ext} and A_{10} were normalized to the intensity of d.l. observed for tris-EDTA washed chloroplast (5 µg/ml) at pH 7.0 10 µs after the flash.

[b] A_{ext} is the initial amplitude of d.l. as obtained from the kinetic analysis.

[c] A_{10} is the d.l. intensity observed 10 µs after laser illumination.

[d] $t_{½}$(fast) is the halflife of the fast phase.

[e] $t_{½}$(slow) is the halflife of the slow phase.

[f] Fraction (fast) is the fraction of the d.l. which decays by the fast phase.

FIGURE III-3

Normalized d.l. decay curves from 7 to 200 μs in dark adapted tris-EDTA washed chloroplasts (5 μg/ml) at the indicated pH. The reaction medium consisted of either 10 m$\underline{M}$ MES (pH 5.2, 6.0) or 10 m$\underline{M}$ HEPES (pH 7.0). Following a single saturating laser flash, a fresh sample was flowed into the cell. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Tris-EDTA washed chloroplasts
Flash 1

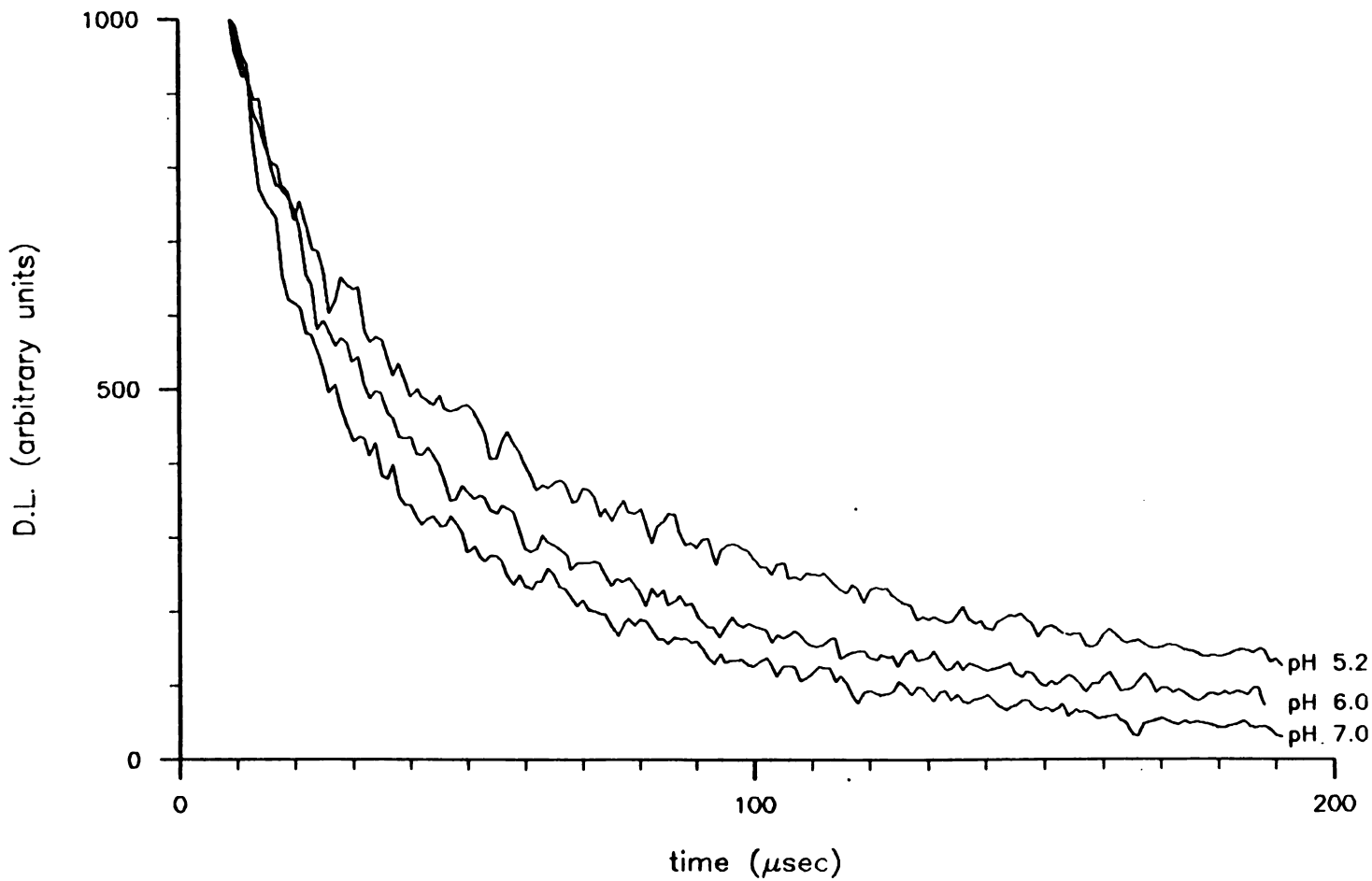
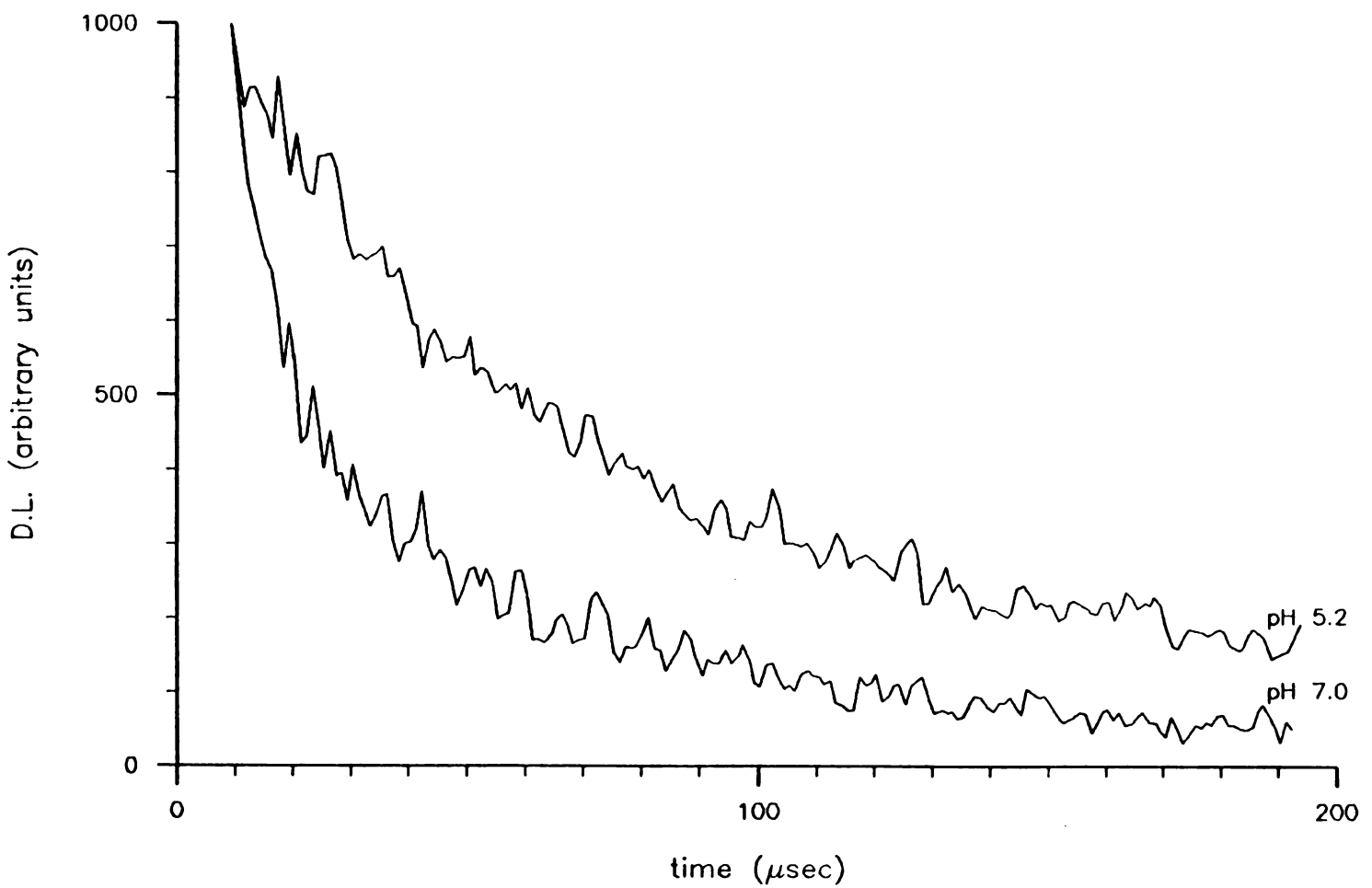


FIGURE III-4

Normalized d.l. decay curves in dark adapted tris-EDTA washed, DCMU treated chloroplasts (5 μg/ml) at the indicated pH values. The reaction media consisted of 10 mM MES (pH 5.2) or 10 mM HEPES (pH 7.0). Following a single saturating laser flash, a fresh sample was flowed into the cell. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Tris–EDTA washed, DCMU treated chloroplasts
Flash 1



phase. The slower phase of d.l. was found to be pH dependent and much faster. The origin of the slower phase of d.l. is unclear; it is perhaps a complicated convolution of several events, all of which are not completely characterized. In terms of the electron transfer model for d.l., there should be included the tendency toward equilibration between P680 and Z^+, the intrinsic back reaction time between $P680^+$ and PQA^-, and the rate of PQA^- oxidation by PQB. The role of mediating effects such as the dynamic aspects of the membrane potential associated with electron transfer is indicated by the fact that this slower phase cannot be adequately described in terms of published values for rate constants and characterized intermediates associated with PSII (see Chapter 5 for a comparison of an electron transfer model for d.l. in tris-DCMU and tris-treated chloroplasts). The possible significance of this slower phase of d.l. is indicated by the light saturation properties of d.l. Figure III-5 shows the extrapolated intensity obtained from a kinetic analysis for the total d.l. (o), the amplitude of the fast phase (+), and the amplitude of the slow phase (*) as a function of light intensity. The identical light saturation behavior demonstrates a strong correlation between the two observed phases of d.l. and rules out the role of the heterogeneous population of primary stable acceptors which were proposed to describe the kinetic behavior of fluorescence induction [85,86]. D.l. measured

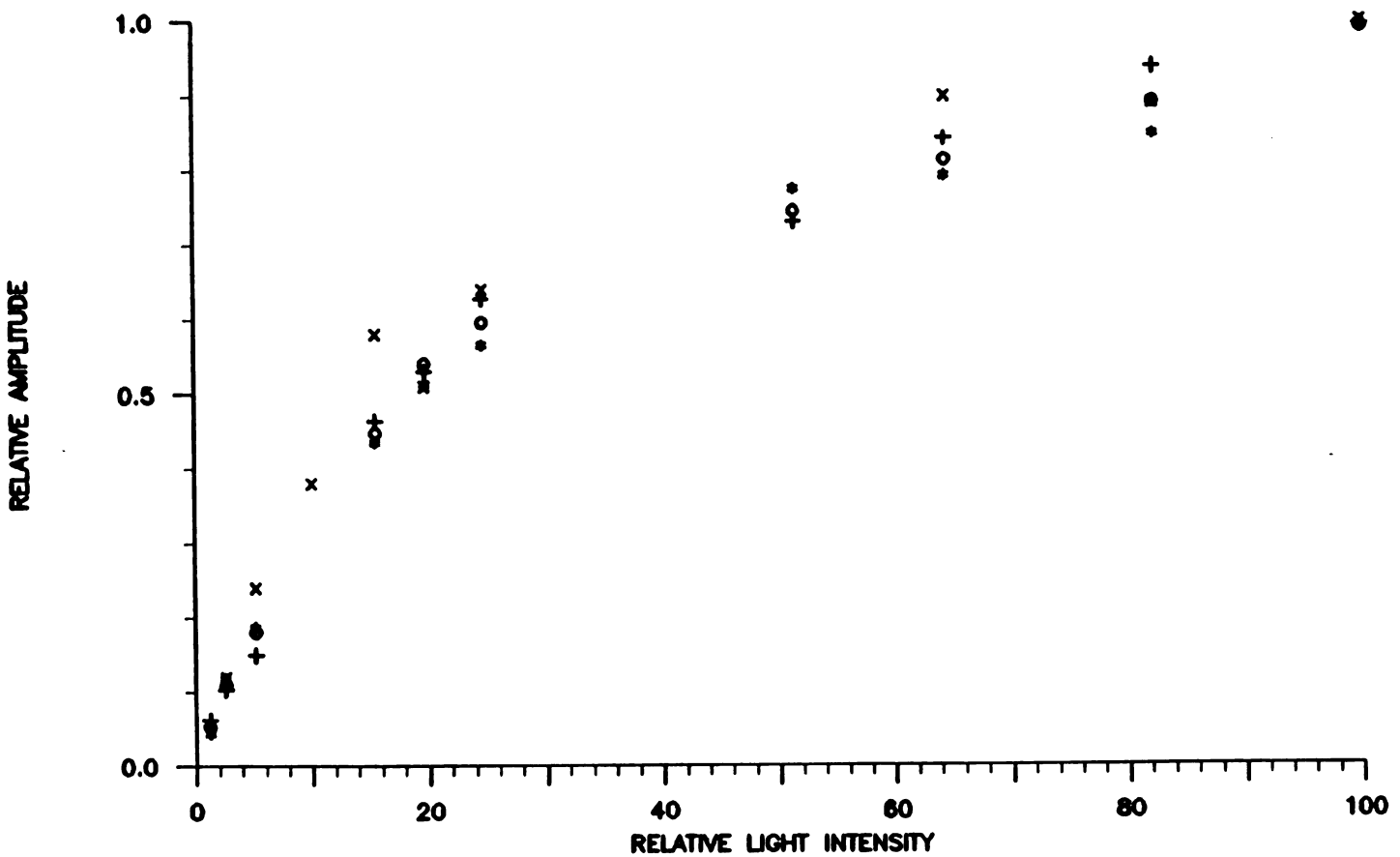
FIGURE III-5

D.l. and induced d.l. light saturation behavior in dark adapted tris-EDTA washed chloroplasts (pH 7.0). Following a single laser flash, a fresh sample was flowed into the cell.

- o Total relative extrapolated d.l. intensity
- \+ Relative intensity of the 7 μs phase
- * Relative intensity of the 50 μs phase
- x Relative intensity of the luminescence induced by an external 900 V/cm electric field

D.L. & EPL LIGHT SATURATION

Tris–EDTA washed chloroplasts
pH 7.0, Flash 1



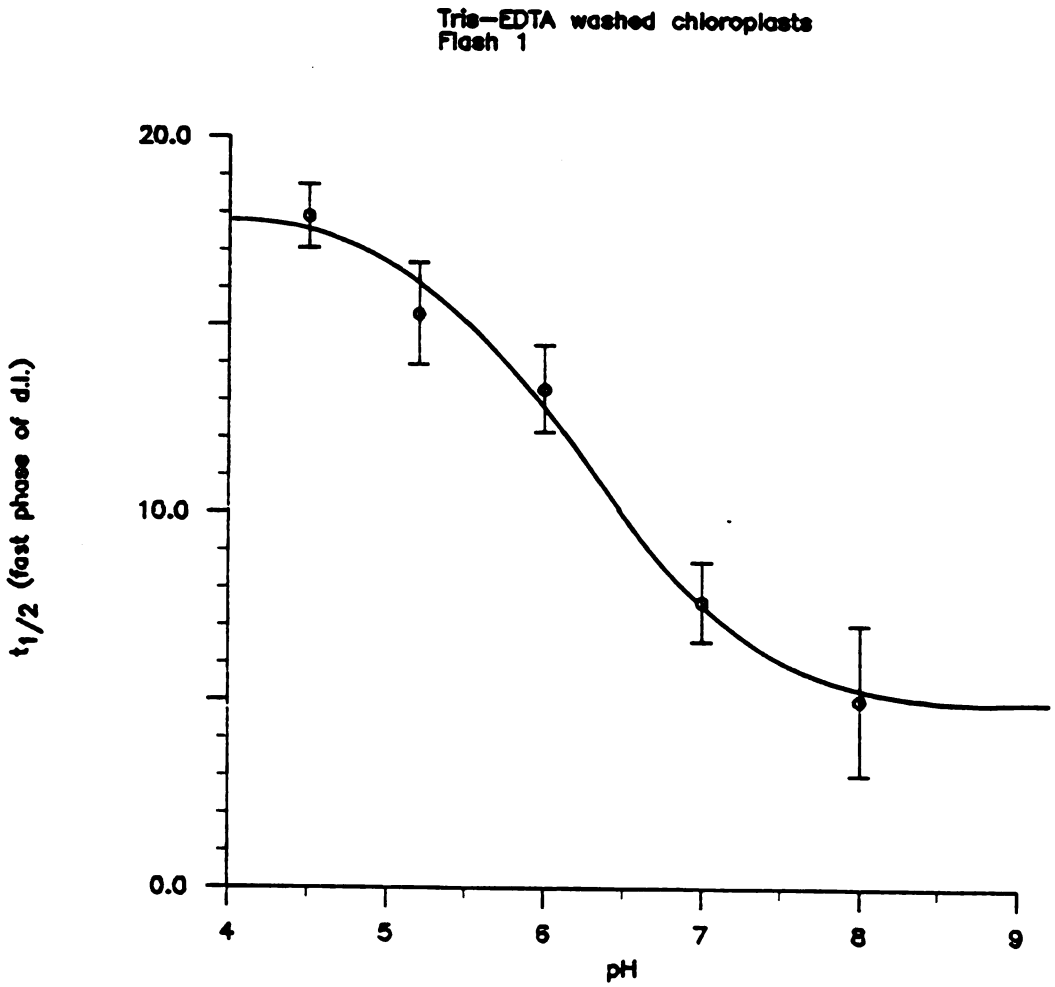
from chloroplasts suspended in a reaction media consisting of 0.4 M sucrose, 10 mM NaCl, and 50 mM buffer displayed a similar pH dependent behavior. Table 2 summarizes the kinetic behavior of d.1. in dark adapted tris-washed chloroplasts. In Table 2, the extrapolated intensity was obtained as the sum of the amplitude of the fast phase and the slow phase.

The pH dependent kinetics of d.1. could possibly provide a probe to the effect of protonation/deprotonation reactions on PSII electron carriers. It might appear that the secondary donor (Z) can exist in either a protonated or deprotonated state with a characteristic reaction rate in each state. The observed rate would then be a convolution of the two states. This model can adequately explain the observed behavior within experimental uncertainty if one assumes a pK_a of about 6.2 and reaction rates corresponding to a halflife of 17.9 and 5.0 μs in the protonated and deprotonated form respectively (Fig. III-6). Since there is evidence that Z is a plastoquinone species [87], the observed rates might represent the equilibrium between the protonated and deprotonated form of a semihydroquinone free radical. Although the pH dependent rate of reduction of $P680^+$ (in the range of pH 4.5 to pH 8.0) appears to be related to the acid-base equilibrium of the electron donor, the kinetics at pH 9.0 continue to change [31]. This indicates that the effect is due to more than a simple protonation of Z. Furthermore, the shape of the

FIGURE III-6

Plot of the fast phase (halflife) of d.l. as a function of pH. The halflives were obtained from the kinetic analysis with the error bars representing the standard deviations of the results of at least 3 experiments. The smooth curve depicts the predicted behavior for $P680^+$ reduction by the simple protonation/deprotonation model for Z. See text for details.

$t_{1/2}$ (fast phase of d.l.) vs. pH



ESR Signal IIf, attributed to Z^+, the oxidized form of the P680 donor, is independent of pH (Figure III-7). In Figure III-7, Signal IIf was measured under steady state conditions with constant illumination. If the pK_a of the oxidized form of Z is outside the pH range studied (4.5 to 8.0), the lineshape should be invariant with pH. The initial amplitudes of the time resolved Signal IIf traces, however, were also independent of pH [88]. This indicates that, at least within the ESR response time (about 10 μs), Z does not deprotonate following oxidation. Thus there does not exist direct evidence that the pH dependent kinetic behavior of d.1. is due to a direct protonation/deprotonation of Z.

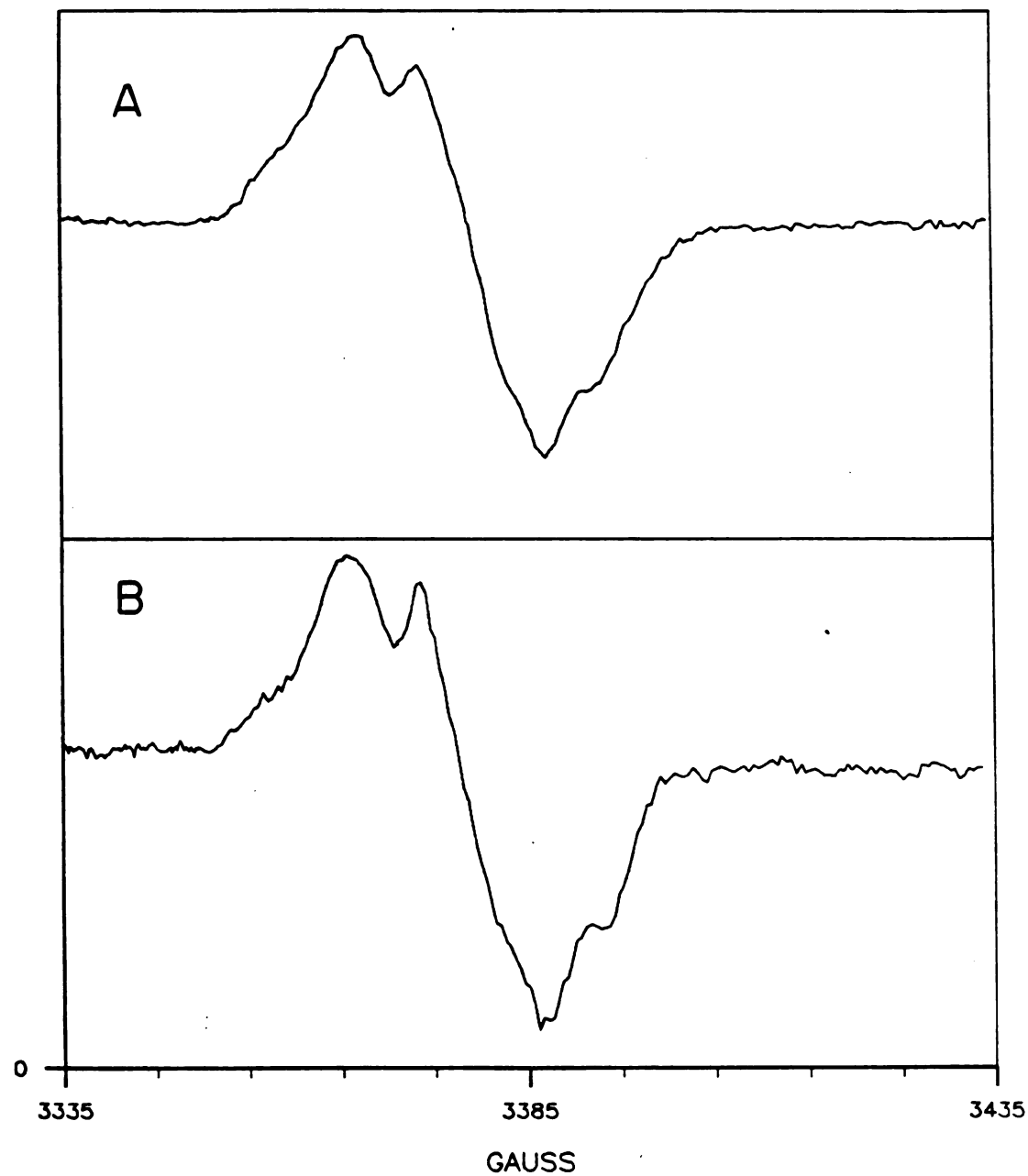
2. Effect of Ionic Strength

Based upon the efficiency of charged electron donors and acceptors to reduce Z^+ or oxidize PQ_A^-, the membrane potential was estimated to be 20 mV (negative inside) at pH 8.0 [89]; the membrane potential arises mainly from differences in the surface charge density of the inside and outside surfaces. The surface charge density, which arises primarily from the protonation and deprotonation of surface charged groups, should be strongly pH dependent. Since the composition of the outer membrane surface differs from the inside surface the membrane potential should also be pH dependent. Although there does not exist a systematic study of the membrane potential in this pH range (4.5 to 8.0),

Figure III-7

Signal IIf in tris-EDTA washed enriched PSII fragments induced by continuous illumination. Curve A) pH 8.0; Curve B) pH 5.2. The signal intensity is normalized for differences in the chlorophyll concentration between the two samples.

SIGNAL II_f



the mediating role of the membrane potential on $P680^+$ reduction can be studied by monitoring the fast phase of d.l. at various ionic strengths. The addition of salt should serve to neutralize the surface charge. The addition of the ionophore gramicidin should facilitate this effect by allowing a rapid diffusion of ions to the inside of the membrane. It was found that the kinetic behavior of d.l. was insensitive to the ionic strength in the presence or absence of gramicidin; the intensity of d.l., however, did vary. The normalized results for various concentrations of KCl and $CaCl_2$ at pH 7.0 and 5.2 are shown in Figures III-8, III-9, and III-10. This demonstrates that the primary reactions of PSII are not affected by the surface charge of the membrane and particularly that the fast phase of $P680^+$ reduction as monitored by d.l. does not depend upon pH dependent membrane potential phenomena.

The amplitude of d.l., however, was extremely sensitive to salt and gramicidin effects. This was particularly true for divalent cations. A 100 m$\underline{M}$ Ca^{2+} concentration resulted in a 40% quenching of the d.l. intensity, while 1 $\underline{M}$ Ca^{2+} essentially quenched d.l. completely (data not shown). Alternatively, identical concentrations of monovalent cations (K^+) caused changes of less than 10% in the d.l. yield. In all cases, gramicidin resulted in a 25 to 40% quenching of d.l. compared to samples which, other than the addition of gramicidin, were treated in an identical manner. These experiments emphasize that under certain conditions,

FIGURE III-8

Normalized d.l. decay curves for dark adapted tris-EDTA washed chloroplasts (5 µg/ml) at pH 5.2 and pH 7.0) as a function of the indicated KCl concentration. In addition to salt, the reaction media consisted of either 10 m<u>M</u> MES (pH 5.2) or 10 m<u>M</u> HEPES (pH 7.0). The amplitude of the signal varied by less than 10% in the range of 0 to 1 <u>M</u> KCl. Following a single saturating laser flash, a fresh sample was flowed into the cell. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Tris–EDTA washed chloroplasts
Flash 1

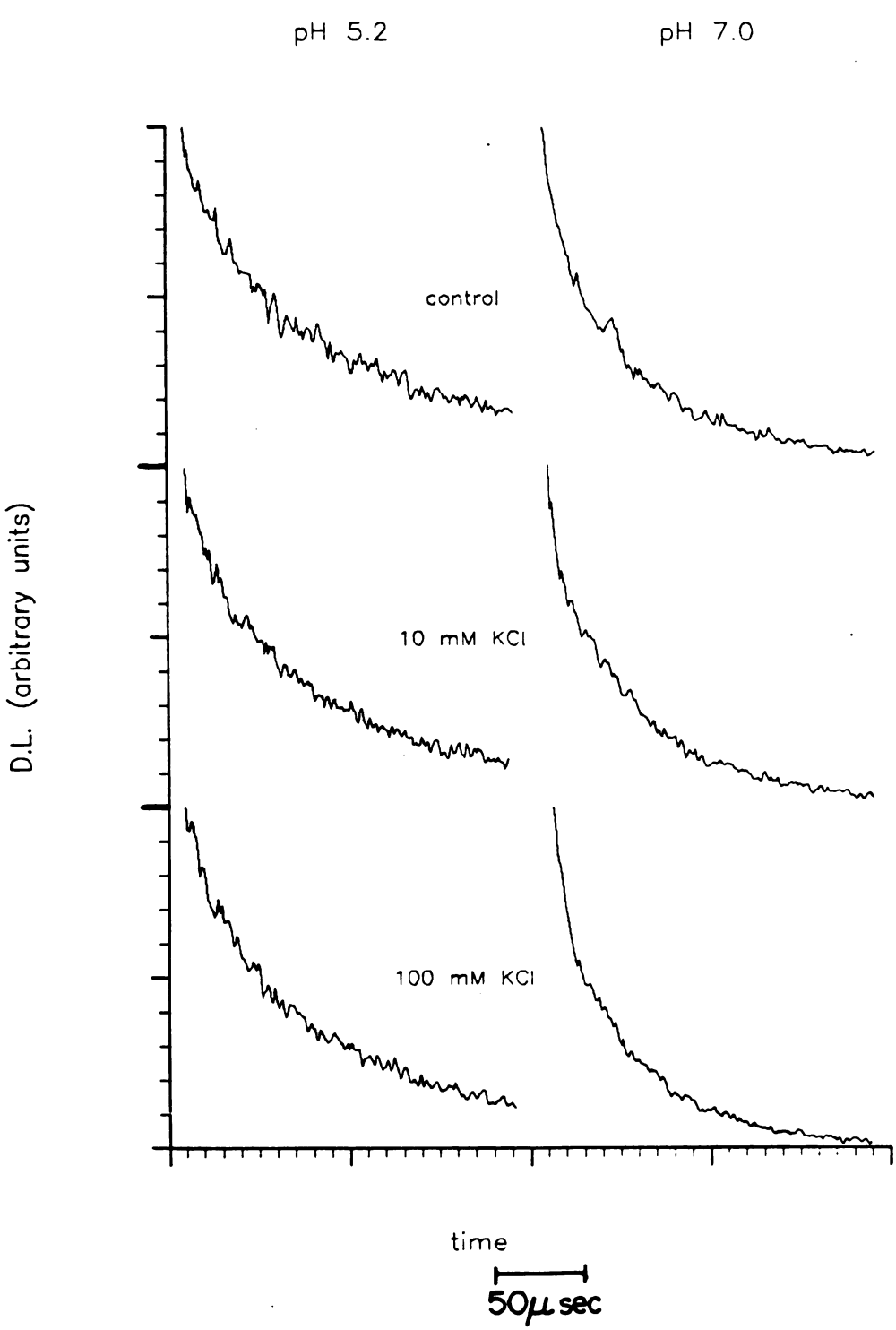


FIGURE III-9

Same conditions as Figure III-8 except that $CaCl_2$ was used instead of KCl. The initial amplitude of d.l. in the presence of 100 m$\underline{M}$ $CaCl_2$ was about 60% of that observed in the presence of 0 or 10 m$\underline{M}$ $CaCl_2$. 1 $\underline{M}$ $CaCl_2$ quenched d.l. nearly 100% (data not shown).

DELAYED LUMINESCENCE

Tris–EDTA washed chloroplasts
Flash 1

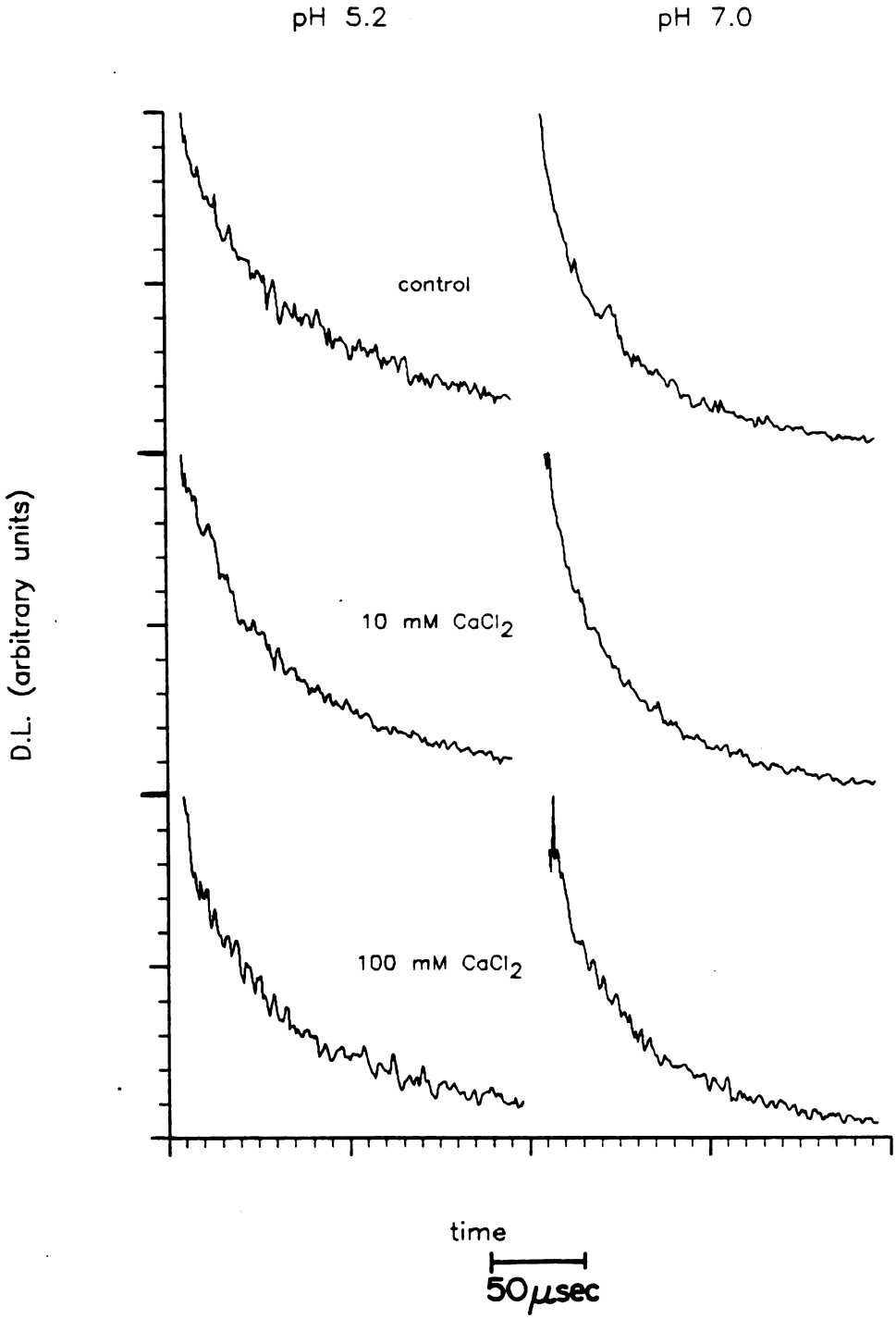
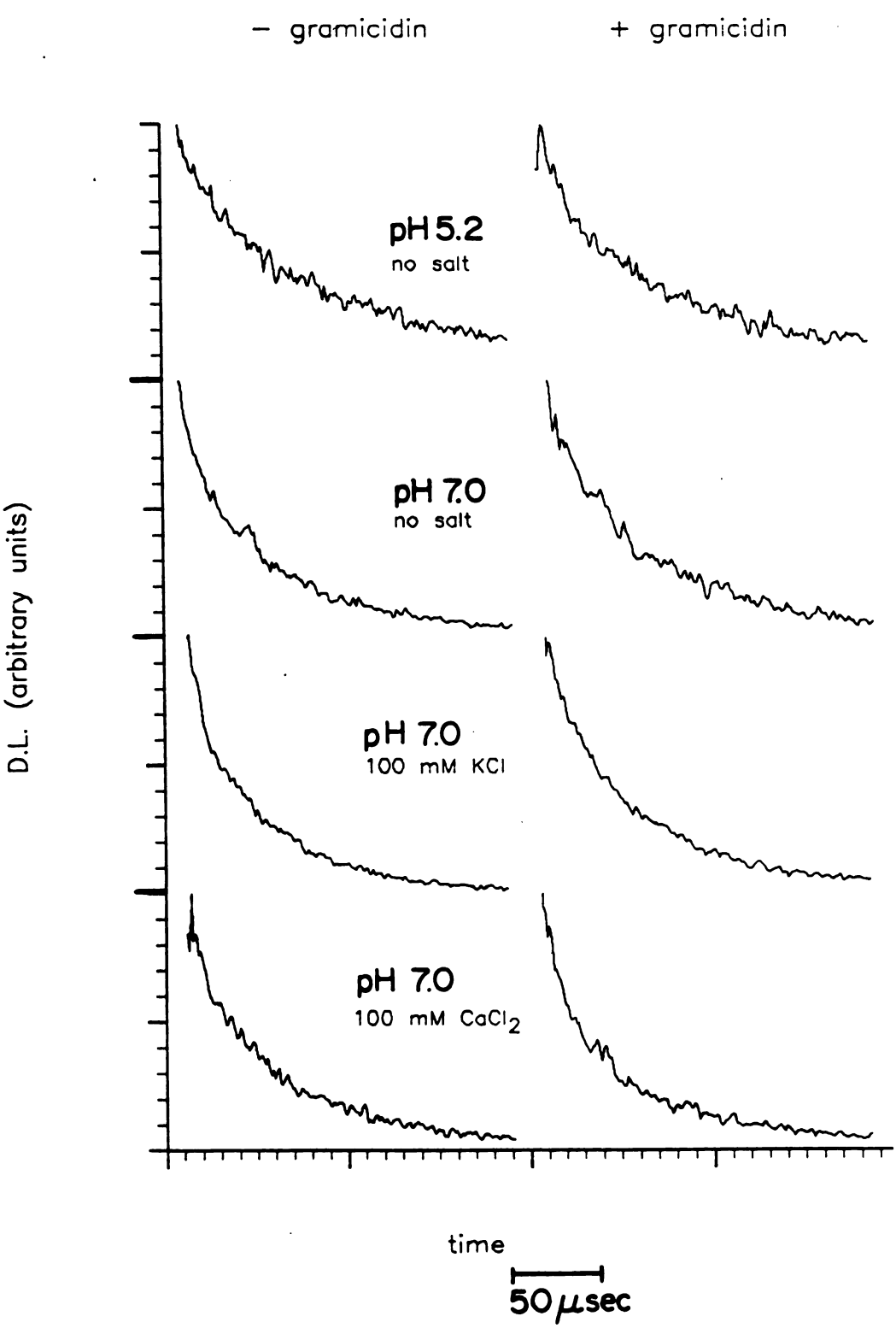


FIGURE III-10

Normalized d.l. decay curves in dark adapted tris-EDTA washed chloroplasts (5 μg/ml) in the presence or absence of gramicidin at the indicated pH and salt concentration. Following a single saturating laser flash, a fresh sample was flowed into the cell. The initial d.l. intensity in the presence of gramicidin was 50 to 75% of the control. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Tris–EDTA washed chloroplasts
Flash 1



the overall luminescence yield can be changed without causing a significant alteration in the kinetics of PSII electron transfer reactions.

3. D.l. from Dark Adapted Untreated Chloroplasts

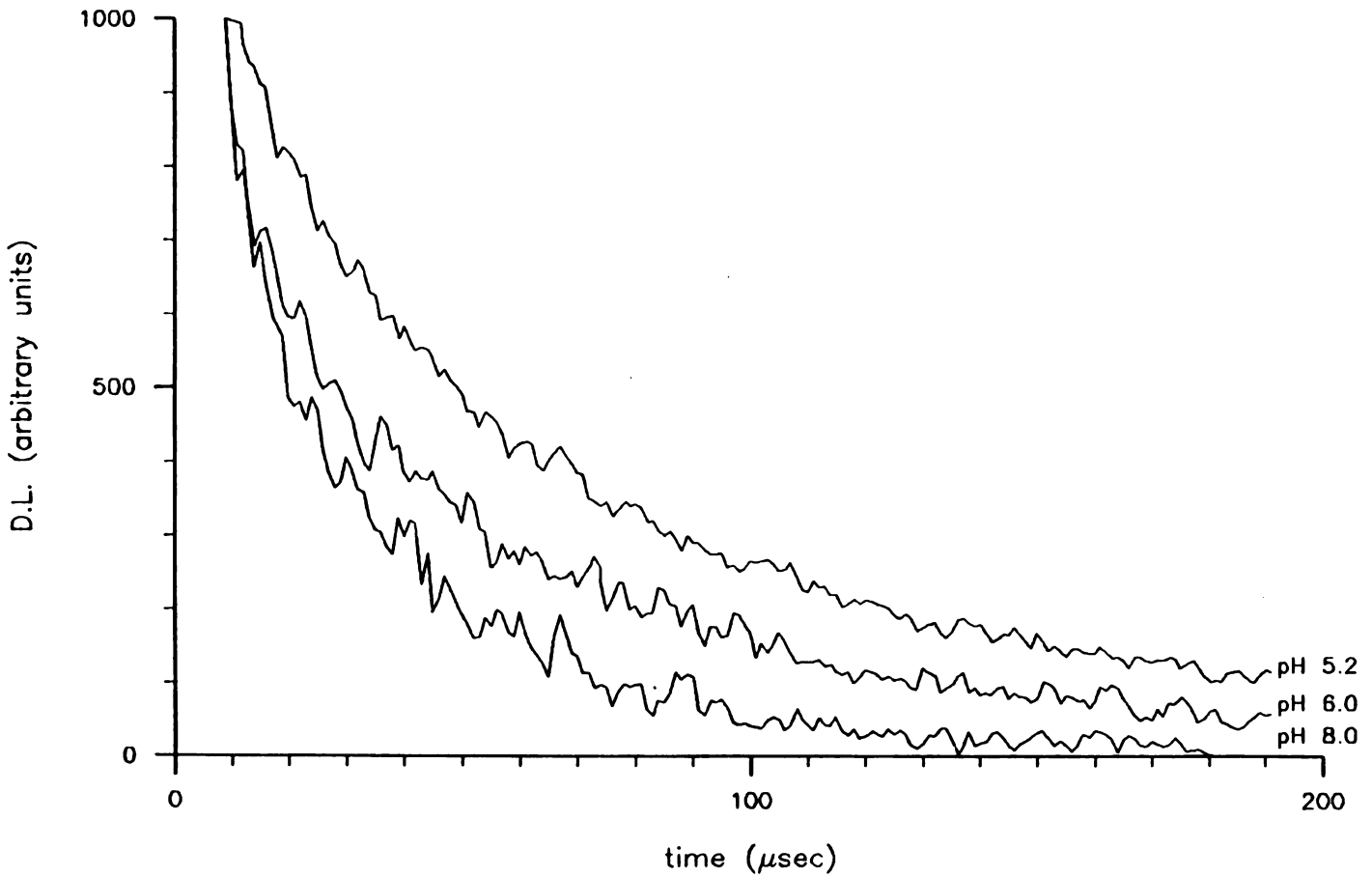
The delayed luminescence in the 7 to 200 μs range from untreated chloroplasts also follows a biphasic pH dependent decay similar to that observed for tris-washed chloroplasts. The results for untreated chloroplasts are summarized in Table 2 and normalized data are shown in Figure III-11. According to present models, $P680^+$ reduction in untreated, dark-adapted chloroplasts should proceed with a 30 ns halflife [25]. There should be no 10 μs phase for d.l. It has been reported, however, that a fraction of $P680^+$ decayed with a 10 μs halflife and that the amplitude of the 10 μs phase at pH 7 is about 1.8 times greater in tris-washed chloroplasts than in untreated chloroplasts [36]. Subsequent investigations have shown, however, that the 10 μs phase represents only a small fraction of the total $P680^+$ reduction [34]. The amplitudes for d.l. summarized in Table 2 show a similar enhanced 10 μs phase for tris inhibited chloroplasts relative to untreated samples. This indicates that the 10 μs phase which is independently observable under different experimental conditions may be of similar origin for both tris and untreated samples.

FIGURE III-11

Normalized d.l. curves for dark adapted untreated chloroplasts (5 μg/ml) at the indicated pH. The reaction media consisted of either 10 m$\underline{M}$ MES (pH 5.2, 6.0) or 10 m$\underline{M}$ HEPES (pH 8.0). Following a single saturating laser flash, a fresh sample was flowed into the cell. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Untreated chloroplasts
Flash 1



4. Effects of Multiple Turnovers

In contrast to DCMU treated tris-washed chloroplasts, d.l. intensity increases with flash number in tris-EDTA inhibited chloroplasts. This enhanced luminescence may be attributed to an increase in the fraction of centers which relax via a radiative back reaction. This effect is a result of an increase in the number of centers with Z oxidized. This can be easily checked by varying the frequency of flash illumination (see Figure III-12A, B, C, and D). The intensity of d.l. for tris-washed chloroplasts following multiple turnovers was found to increase with increasing flash repetition rate. An analysis of the intensity of d.l. following flash number 2 or 3 relative to the d.l. intensity following flash number 1 indicated that the sample relaxed with a halflife of about 2.6 seconds (Figure III-13). Essentially the same dark relaxation time was observed for the dark decay of Z^+ as monitored by the dark decay of the ESR Signal IIf [79]. It has been reported that the halflife of the slower phase of d.l. increases as the flash repetition rate increases [83]. In contrast, the kinetic phases of the data shown in Figure III-12 were invariant with the rate of illumination. The results of Figure III-12 were obtained from each of four actinic light pulses, whereas in [83], d.l. was measured from samples preilluminated by an unspecified number of flashes. The addition of 0.3 m$\underline{M}$ phenylenediamine (Pd) (plus 2.0 m$\underline{M}$ ascorbate), an exogenous reductant which serves as an

FIGURE III-12

D.l. following each of 4 actinic flashes in dark adapted tris-EDTA washed chloroplasts (5 μg/ml) in 10 mM HEPES (pH 7.0) normalized to the initial d.l. intensity induced by the first flash. Curves A, B, C, and D no additions. Curves E and F plus 0.3 mM phenylenediamine/2.0 mM ascorbate. Flash repetition rate: 0.5 Hz curve A, 1 Hz curves B and E, 4 Hz curve C and F, 10 Hz curve D. Following the fourth flash, a fresh sample was flowed into the sample cell. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Tris–EDTA washed chloroplasts
pH 7.0

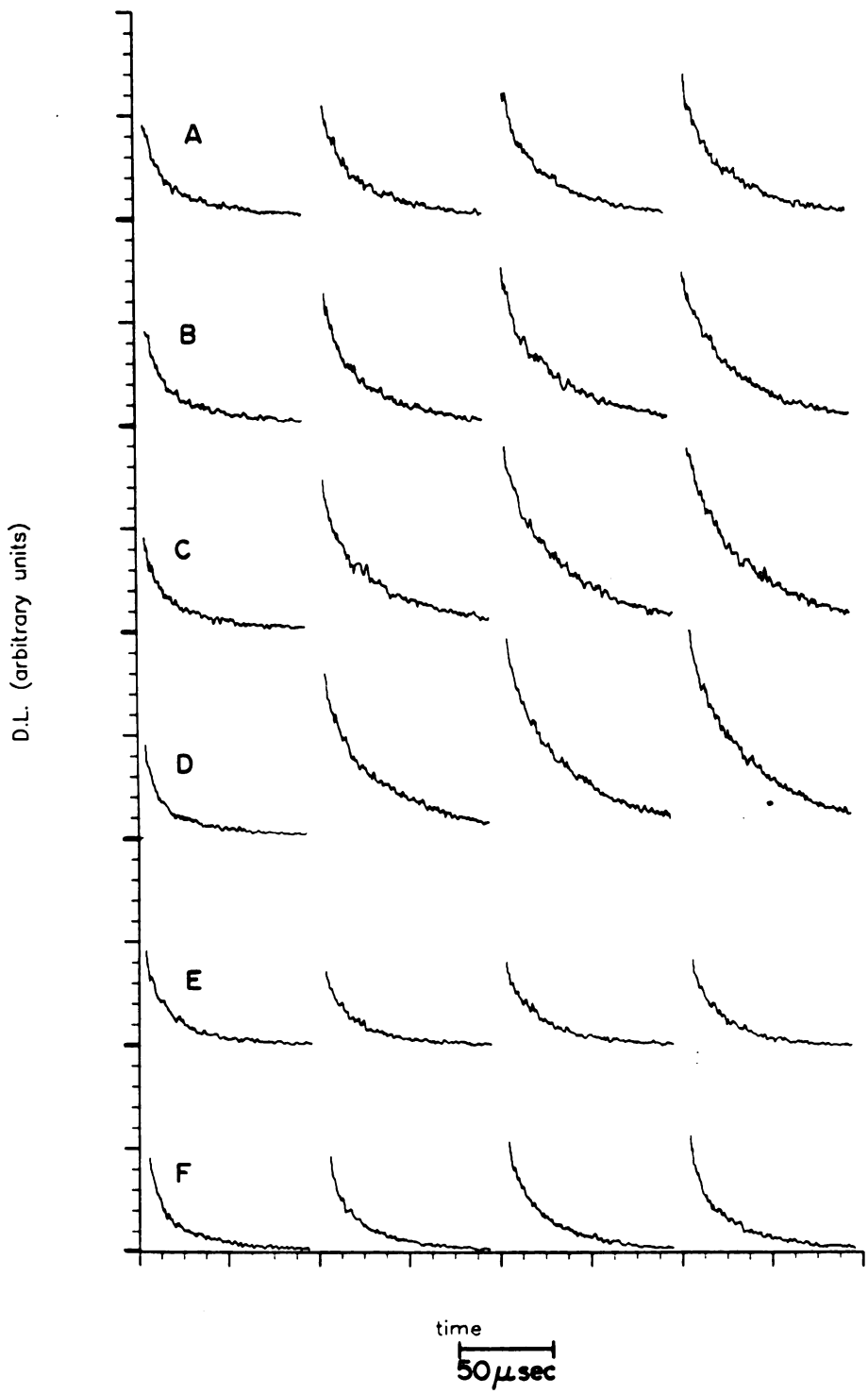
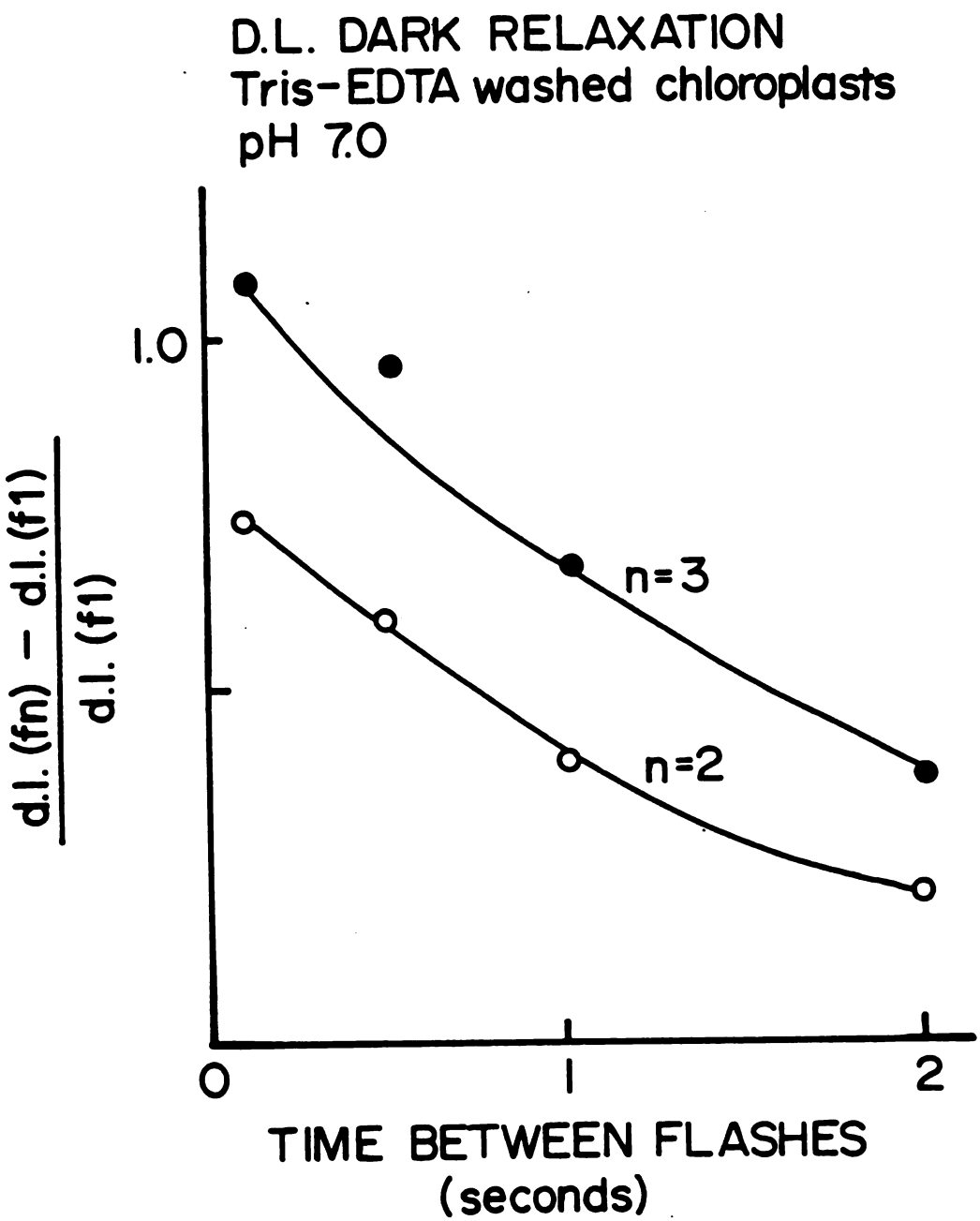


FIGURE III-13

Plot of the difference of the initial d.l. intensity in tris-EDTA washed chloroplast (pH 7.0) induced by flash number 2 and 3 relative to that induced by a single flash 1 and normalized to the d.l. intensity induced by the first flash: $[dl(f_n) - dl(f_1)]/[dl(f_1)]$; $n = 2,3$ as a function of dark time between flashes.


D.L. DARK RELAXATION
Tris-EDTA washed chloroplasts
pH 7.0
1.0
d.l. (fn) − d.l. (f1) / d.l. (f1)
n=3
n=2
0
1
2
TIME BETWEEN FLASHES
(seconds)

electron donor to Z^+, enhances the rate of dark relaxation of PSII. At a flash repetition rate of 1 Hz, the d.l. intensity was flash number independent. The flashing frequency had to be increased to greater than 4 Hz before any enhancement of d.l. was observed. The relative enhancement was quenched compared to that obtained without phenylenediamine (Figure III-12E and F). Jursinic and Govindjee [83] found a similar enhanced rate of dark relaxation in tris-washed chloroplasts upon the addition of System II donors. However, these measurements were performed on samples undergoing repetitive flash illumination; there was no comparison to dark adapted chloroplasts. According to the second order rate constant for the phenylenediamine reduction of Z^+ (4.5×10^5 M^{-1} s^{-1}) reported by Babcock and Sauer [79], 0.3 m$\underline{M}$ Pd should reduce Z^+ with a 5 ms halflife. No effect on the decay kinetics of d.l. in the 7 to 200 μs time range in the presence of Pd was observed. This is because the kinetics of Pd effects are much slower than the time scale studied for d.l. In the following experiments, the standard flash repetition rate was 1 Hz.

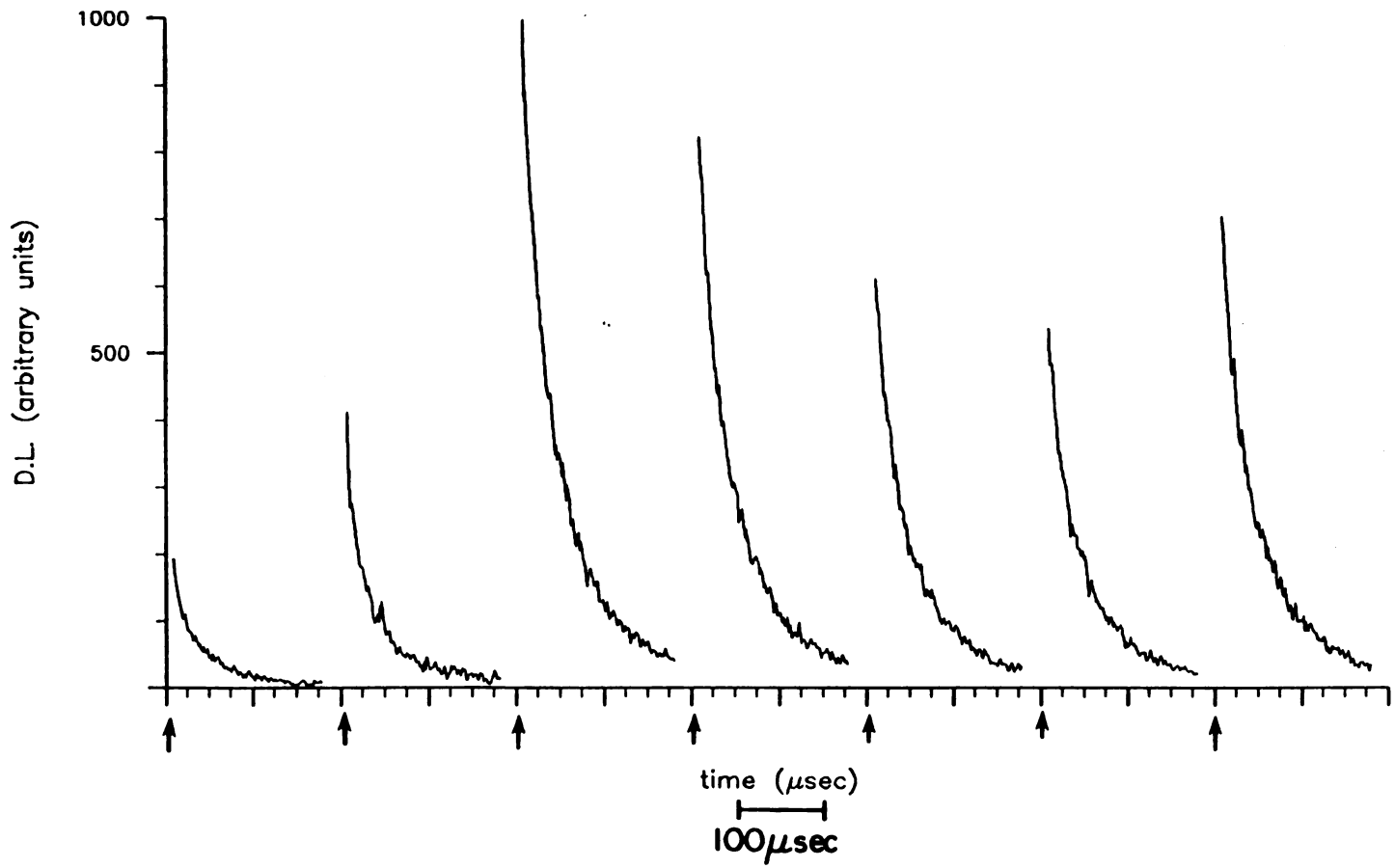
In untreated chloroplasts, d.l. follows the same four flash oscillatory behavior as that observed for O_2 evolution as shown in Figure III-14. The existence of the flash oscillations indicates that the d.l. which emanates from dark adapted samples may originate, at least in part, from

FIGURE III-14

Relative d.l. intensity following each of 7 actinic flashes in untreated chloroplasts (5 μg/ml) in 10 m*M* HEPES (pH 7.0). The flash repetition rate was 1 Hz. The arrow indicates the time of illumination. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Untreated chloroplasts
pH 7.0



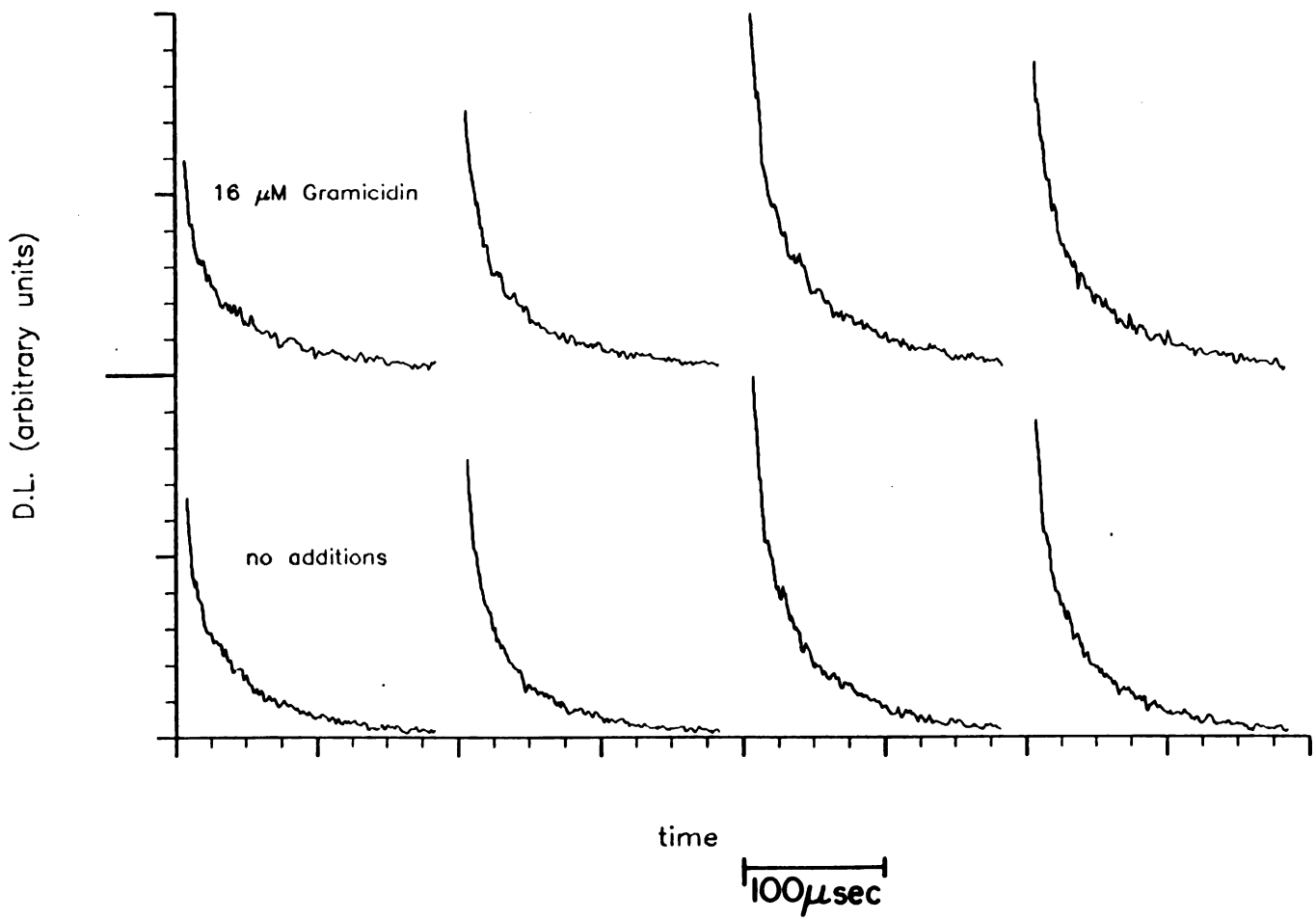
intact centers. If the 10 μs phase observed in untreated samples originates from damaged centers, flash oscillatory behavior would not be expected. However, changes in the membrane potential arising from an accumulation of charge following multiple turnovers cannot be completely neglected. For example, it is now generally accepted that proton release associated with water oxidation does not occur in a concerted reaction. Rather it occurs sequentially as oxidation equivalents are stored. The proton release to the aqueous phase displays a stoichiometry of 1:0:1:2 for the transitions $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4$ respectively [90]. Thus for dark adapted chloroplasts with 25% of the centers in the S_0 state and 75% in the S_1 state, there should be a maximum charge stored on the oxidizing side of PSII just prior to the third flash. The four flash oscillatory behavior is easily observed in a reaction media consisting of 0.4 M sucrose, 50 mM HEPES (pH 7.5), and 10 mM KCl and shows a maximum intensity on the third flash (see Figure III-15). The addition of gramicidin did not have an affect on the kinetics or relative intensity with flash number of d.l.; the absolute intensity of d.l. in the presence of gramicidin was about 85% of the control. This eliminates the possibility that changes in the bulk transmembrane potential are the cause for the flash induced oscillation of d.l. However, local fields, such as those caused by the storage of charge, may not be affected by gramicidin. This is evidenced by the four flash

FIGURE III-15

D.l. following each of 4 actinic flashes for untreated chloroplasts (5 μg/ml) suspended in 0.4 M sucrose, 50 mM HEPES (pH 7.5), and 10 mM KCl (SHK) in the presence and absence of gramicidin. The intensity of d.l. in the presence of gramicidin was about 85% of the control. The flash repetition rate was 1 flash/second. Following the fourth flash, a fresh sample was flowed into the cell. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Untreated chloroplasts
pH 7.5 in SHK



oscillatory behavior of d.l. even after approximately 35% of the PSII centers were damaged as measured by changes in the steady state oxygen yield (see Figure III-16). The d.l. measurements shown in Figure III-16 were obtained from a sample of chloroplasts which were inhibited by a mild heating procedure. Figure III-17 shows the effect of increasing exposure time in a 50°C water bath on rates of oxygen evolution and the initial intensity of d.l. induced by the first and third actinic light flashes normalized to the sum of the initial d.l. yield induced by the first four flashes. It is apparent that the heating procedure has an immediate effect on oxygen yields, but there exists an induction time before any effect is observed on d.l. However, as mentioned, the oxygen measurements were performed under steady state conditions. It is therefore not definitively established that the heat induced inhibition is due to effects on the oxidizing side of PSII, although this is the most likely inhibition site for mild heat treatments [39].

As reported by Bowes and Crofts [82], the flash oscillatory behavior for d.l. is observed around pH 7.0, becoming quenched at pH less than 6.0 and pH greater than 8.0. The d.l. flash number dependence for untreated chloroplasts is shown in Figure III-18 and is summarized in Table 3. A similar summary for the flash number dependence of d.l. in tris-washed chloroplasts is in Figure III-19 and summarized in Table 4. Most notable in

FIGURE III-16

D.1. following each of 4 actinic flashes in untreated chloroplasts (5 µg/ml) exposed to a 50°C water bath for 30 seconds which resulted in a 35% inhibition of the rate of steady state oxygen evolution. The reaction media was 10 m<u>M</u> HEPES (pH 7.0). Following the fourth flash a fresh sample was flowed into the cell. Each trace is the average of 10 measurements.

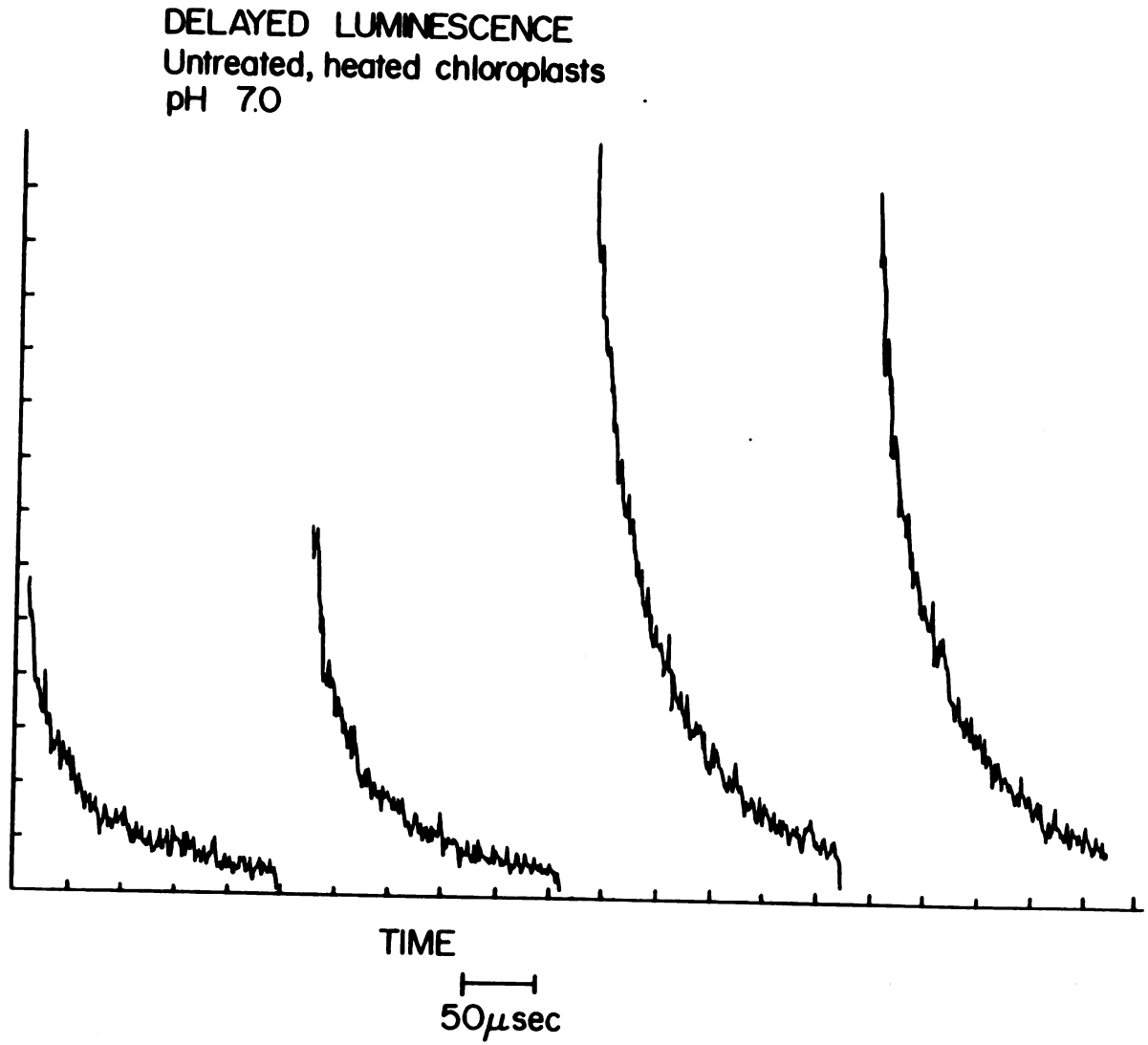

DELAYED LUMINESCENCE
Untreated, heated chloroplasts
pH 7.0
TIME
50μsec

FIGURE III-17

Plot of the effect of mild heating (50°C) on d.l. behavior and steady state oxygen evolution. The d.l. result is plotted as the initial d.l. intensity induced by flash 1 and 3 normalized to the sum of the initial d.l. intensity induced by flash 1 through 4. The control rate of oxygen evolution was 225 μmole O_2/mg_{Chl}-hr.

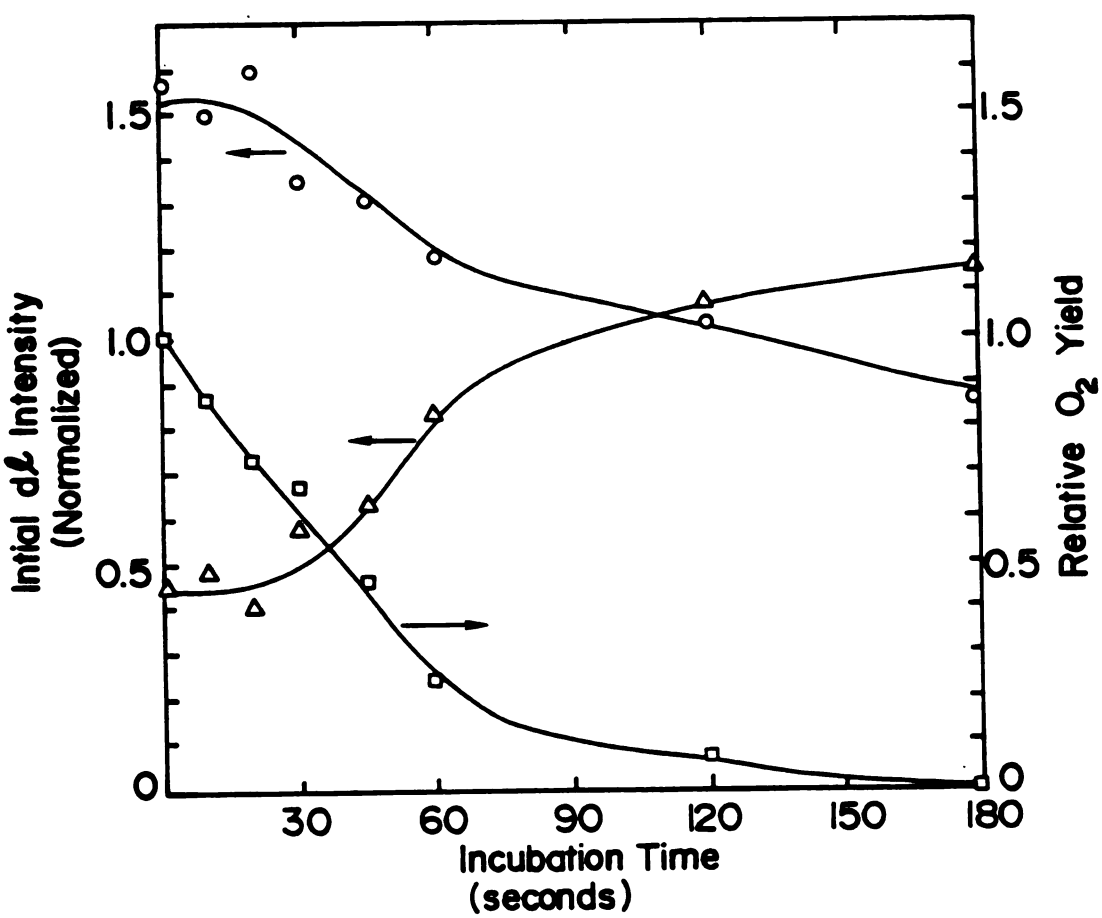

Intial dℓ Intensity
(Normalized)
1.5
1.0
0.5
0
30
60
90
120
150
180
Incubation Time
(seconds)
Relative O_2 Yield

FIGURE III-18

D.l. curves induced by each of 4 actinic flashes in untreated chloroplasts (5 μg/ml) at the indicated pH and normalized to the maximal observed intensity. The reaction media consisted of either 10 m<u>M</u> succinate (pH 4.5), 10 m<u>M</u> MES (pH 5.2, 6.0), or 10 m<u>M</u> HEPES (pH 7.0, 8.0). Following the fourth flash, a fresh sample was flowed into the cell. For pH 7.0 and 6.0, the maximum d.l. intensity was induced by flash 3. At pH 4.5, 5.2, and 8.0, the d.l. intensity was maximal on the fourth flash. Each trace is the average of 10 measurements.

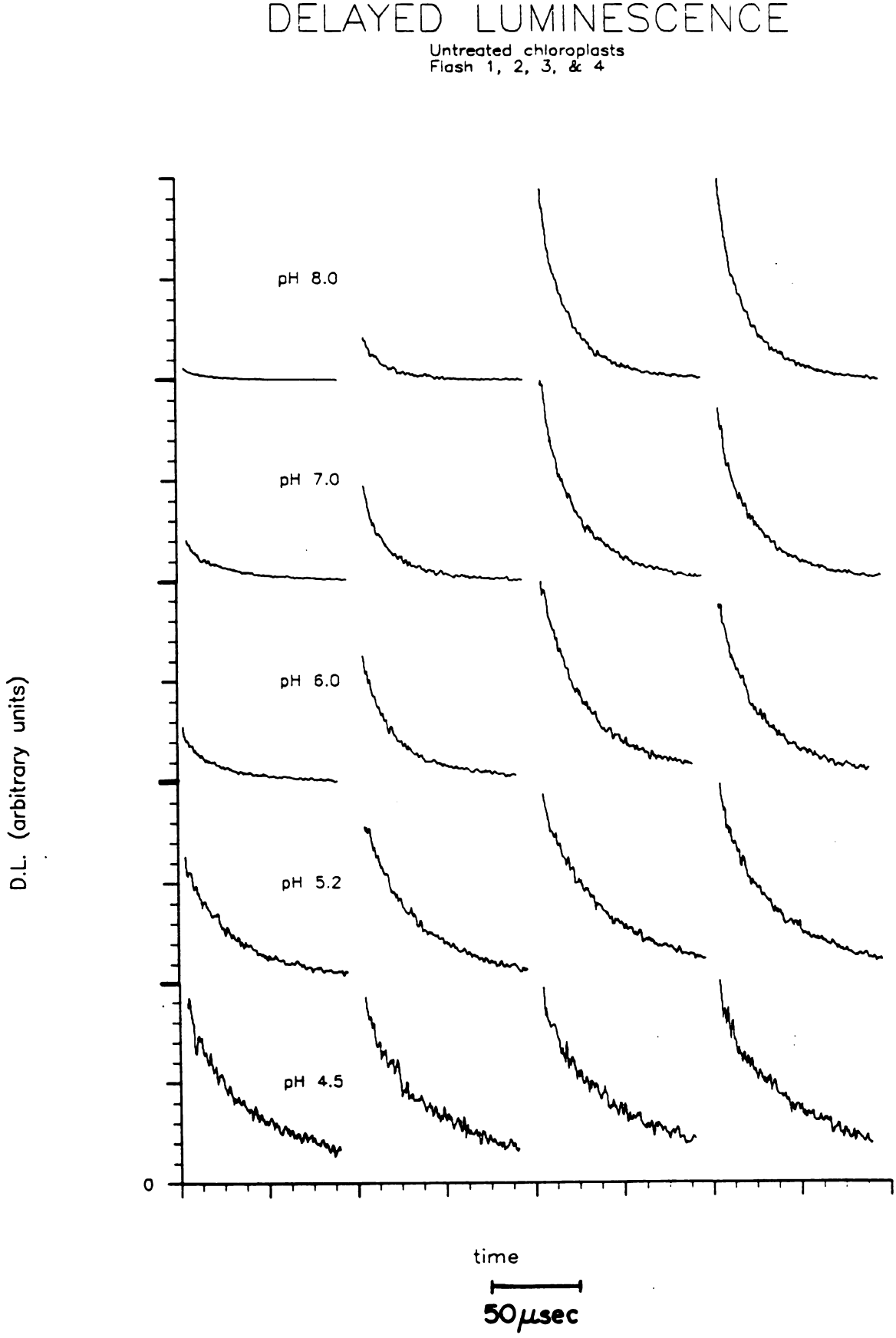

DELAYED LUMINESCENCE
Untreated chloroplasts
Flash 1, 2, 3, & 4
pH 8.0
pH 7.0
pH 6.0
pH 5.2
pH 4.5
D.L. (arbitrary units)
0
time
50μsec

TABLE 3 Delayed Luminescence Kinetic Behavior.[a]

Untreated		A_{ext}[b]	A_{10}[c]	$t_{½}$(fast)[d]	$t_{½}$(slow)[e]	Fraction (fast)[f]
pH 4.5	Flash 1	538 ± 15	448 —	18.3 ± 1.36	89.0 ± 7.5	0.37 —
	Flash 2	571 —	474	15.0 —	91.0 —	0.40 —
	Flash 3	587 —	497 —	13.5 —	98.0 —	0.37 —
	Flash 4	655 —	510 —	9.0 —	70.1 —	0.37 —
pH 5.2	Flash 1	587 ± 33	459 ± 14	17.0 ± 4.9	73.2 ± 7.0	0.53 ± 0.07
	Flash 2	771 ± 91	615 ± 70	22.3 ± 0.7	87.5 ± 24.3	0.53 ± 0.1
	Flash 3	906 ± 94	772 ± 47	19.9 —	86.8 ± 1.8	0.42 ± 0.01
	Flash 4	952 ± 117	794 ± 78	15.0 ± 0.05	80.0 ± 5.0	0.44 ± 0.01
pH 6.0	Flash 1	335 ± 29	211 —	8.9 ± 1.7	48.8 ± 1.0	0.56 ± 0.02
	Flash 2	718 —	518 —	17.8 —	84.1 —	0.80 —
	Flash 3	1088 —	875 —	24.9 —	95.1 —	0.72 —
	Flash 4	1028 —	775 —	19.8 —	68.0 —	0.62 —
pH 7.0	Flash 1	441 ± 77	229 ± 1	7.1 ± 1.7	38.6 ± 4.7	0.65 ± 0.08
	Flash 2	781 ± 209	444 ± 82	9.2 ± 0.2	50.0 ± 7.1	0.67 ± 0.07
	Flash 3	1476 ± 155	1000	12.2 ± 3.2	44.0 ± 5.35	0.55 ± 0.06
	Flash 4	1402 ± 150	871 ± 18	8.3 ± 2.45	39.0 ± 3.45	0.48 ± 0.03
pH 8.0	Flash 1	394 ± 240	148 —	4.9 ± 0.8	52.0 ± 22.0	0.77 ± 0.07
	Flash 2	594 —	259 —	5.7 —	24.6 —	0.64 —
	Flash 3	1813 ± 816	1039 ± 186	9.6 ± 5.0	30.5 ± 7.0	0.55 ± 0.10
	Flash 4	1980 ± 813	1163 ± 466	6.8 ± 0.7	28.0 ± 1.0	0.44 ± 0.02

[a]The uncertainties represent standard deviations. The value listed in the columns A_{ext} and A_{10} were normalized to the d.l. intensity observed 10 μs after the third flash at pH 7.0. The flash repetition rate was 1 flash level.

b-f As in Table 1.

FIGURE III-19

Normalized d.l. decay curves for each of 4 actinic flashes in dark adapted tris-EDTA washed chloroplasts (5 μg/ml) normalized to intensity induced by flash number 4. All other conditions were as described for Figure III-18. Each trace is the average of 10 measurements.

DELAYED LUMINESCENCE

Tris–EDTA washed chloroplasts
Flash 1, 2, 3, & 4

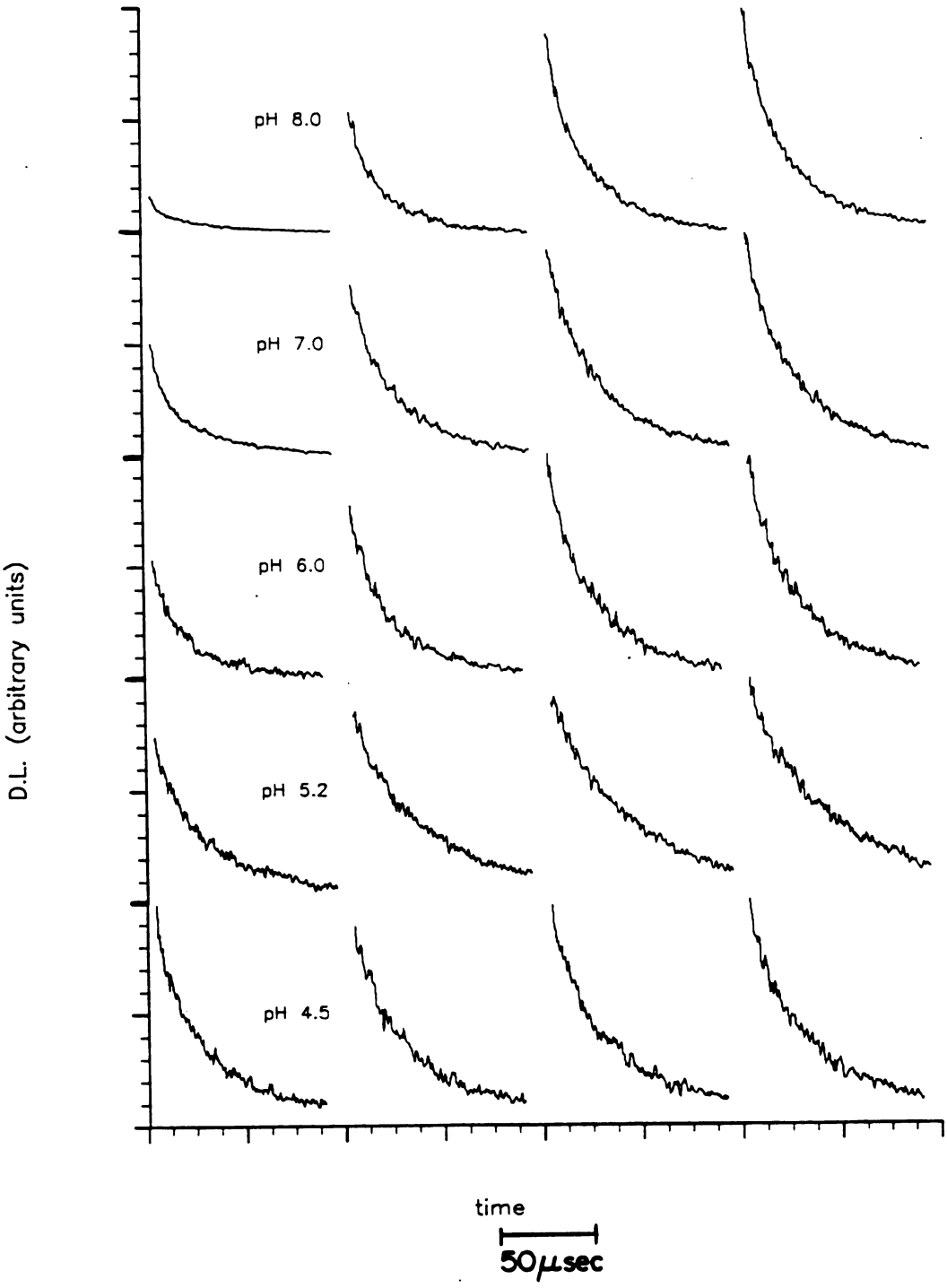


TABLE 4 Delayed Luminescence Kinetic Behavior.[a]

Tris-EDTA washed		A_{ext} [b]	A_{10} [c]	$t_{1/2}$ (fast) [d]	$t_{1/2}$ (slow) [e]	Fraction (fast) [f]
pH 4.5	Flash 1	648 ± 63	516 —	17.9 ± 0.6	79.0 ± 0.82	0.49 ± 0.08
	Flash 2	491 —	469 —	23.2 —	95.2 —	0.73 —
	Flash 3	617 —	514 —	18.0 —	79.1 —	0.53 —
	Flash 4	620 —	506 —	14.9 —	71.9 —	0.45 —
pH 5.2	Flash 1	483 ± 84	373 ± 42	14.9 ± 1.6	76.0 ± 12.1	0.58 ± 0.08
	Flash 2	545 ± 79	444 ± 59	16.4 —	75.8 —	0.35 —
	Flash 3	609 ± 147	494 ± 111	16.8 —	73.4 —	0.22 —
	Flash 4	636 ± 162	540 ± 106	12.7 —	74.0 —	0.26 —
pH 6.0	Flash 1	368 ± 4	267 —	12.6 ± 1.92	66.0 ± 12.9	0.61 ± 0.08
	Flash 2	508 —	389 —	11.4 —	40.4 —	0.65 —
	Flash 3	754 —	500 —	6.7 —	39.8 —	0.38 —
	Flash 4	708 —	481 —	9.3 —	46.1 —	0.37 —
pH 7.0	Flash 1	544 ± 117	307 ± 35	7.6 ± 1.1	45.8 ± 3.9	0.64 ± 0.04
	Flash 2	725 ± 71	486 ± 17	8.6 ± 3.5	45.4 ± 7.3	0.50 ± 0.09
	Flash 3	872 ± 120	602	6.4 ± 0.21	45.6 ± 6.9	0.49 ± 0.01
	Flash 4	1011 ± 149	628 ± 30	8.9 ± 2.9	41.6 ± 1.2	0.40 ± 0.06
pH 8.0	Flash 1	484 ± 314	212 ± 90	5.8 ± 1.3	41.6 ± 8.7	0.73 ± 0.05
	Flash 2	712 ± 79	445 ± 23	7.2 ± 0.55	33.2 ± 1.5	0.55 ± 0.03
	Flash 3	1077 ± 394	631 ± 101	7.9 ± 1.2	38.0 ± 1.1	0.50 ± 0.01
	Flash 4	1128 ± 380	723 ± 106	9.9 ± 1.8	44.2 ± 2.1	0.53 ± 0.01

[a]The uncertainties represent standard deviations. The values listed in the columns A_{ext} and A_{10} were normalized to the d.l. intensity observed in untreated chloroplasts 10 μs after the third flash. The flash repetition rate was 1 flash per second.

b-f
As in Table 1.

the tris-washed samples is the absence of any flash oscillations at any pH. A summary of the amplitudes for flash number 1, 2, 3, and 4 at various pH's is shown in Figure III-20. Several points about Tables 3 and 4 and Figures III-18 through III-20 are apparent. The kinetic phases which show an oscillatory behavior in untreated chloroplasts are present in tris-washed samples. Additionally, even though electron transport is significantly perturbed near the reaction center in tris-washed chloroplasts, the intensity of d.l. differs by less than a factor of two between untreated and tris-washed samples which are otherwise treated in an identical manner. This result will be discussed in Chapter 5.

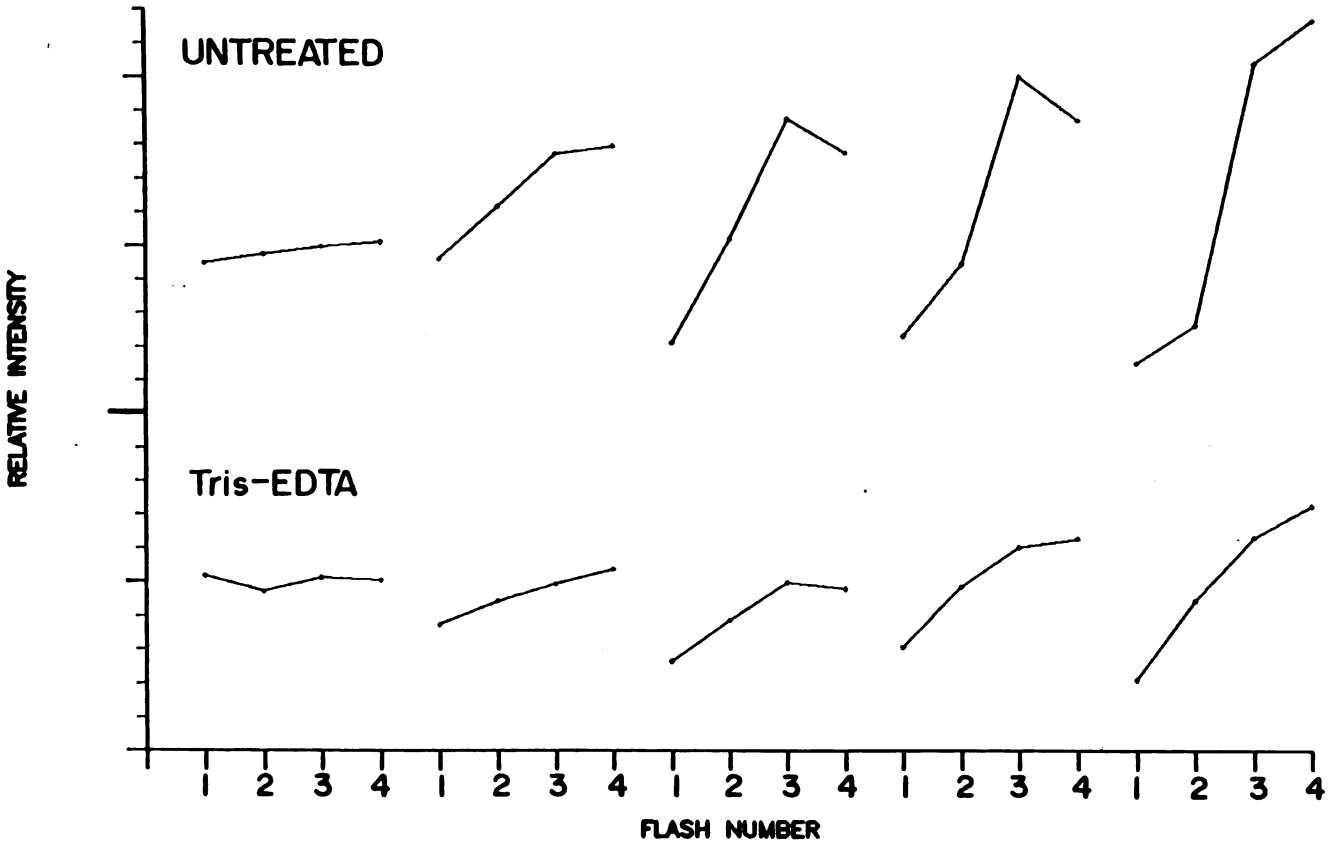
Other than the observation that the d.l. kinetics were pH dependent throughout the range 4.5 to 8.0, the results summarized in Tables 3 and 4 are in general agreement with earlier reports of d.l. in the 6 to 200 μs time range [82,83]. The absorption change associated with the formation of the cationic form of the reaction center of PSII in tris-washed chloroplasts is maximal on the second flash. There are no further enhancements in the magnitude of the absorption change for flash numbers greater than 2 [31]. In these optical studies, $P680^+$ decay was biphasic; in dark adapted samples the fast phase was dominant whereas in samples illuminated by two flashes, the slower phase became dominant. Table 4 shows a similar effect for d.l. behavior. Unlike the optical studies on $P680^+$ decay, the d.l. intensity

FIGURE III-20

Summary of the initial d.l. intensity for untreated and tris-EDTA washed chloroplasts following each of 4 actinic flashes at pH 4.5, 5.2, 6.0, 7.0, and 8.0. The plots are normalized to the d.l. intensity induced by flash 3 at pH 7.0 in untreated chloroplasts.

RELATIVE d.I. INTENSITY vs. FLASH NUMBER

Untreated and Tris–EDTA washed chloroplasts



in tris-washed chloroplats did not become invariant to flash number until the third flash. This is in agreement with the results reported by Jursinic and Govindjee [83]. Additionally, in contrast to the $P680^+$ decay, which has numerous reports for a 100 to 200 μs decay phase [31], d.l. in the 7 to 200 μs time range displayed no evidence for this component. When monitored from 60 to 500 μs so that there would be no contribution from the fast phase, d.l. from dark adapted tris-washed chloroplasts at pH 7.0 decayed monophasically with a halflife of about 65 μs. An identical monophasic decay was observed when the sample was illuminated by two flashes with a dark time of 100 ms between flashes. The slight discrepancy between the 65 μs phase and the values shown in Table 4 most likely originates from differences in the experimental protocol. The results of Table 4 were obtained under conditions which accented the contribution of the fast phase, whereas when d.l. is monitored from 60 to 500 μs, there are, at most, only minor interferences from the rapidly decaying components of d.l. In contrast, at pH 5.2, the d.l. from 60 to 500 μs induced by the second of two light flashes decayed monophasically with a halflife of 130 μs. The dark time between flashes was 100 μs. The reason for the discrepancy between the 130 μs decay time and that of the slower phase listed in Table 4 is probably related to differences in the rate of flash illumination. It is not clear why a similar effect of

flash repetition rate on d.l. kinetics was not observed at pH 7.0.

CHAPTER 4

ELECTROPHOTOLUMINESCENCE

A. Overview of Electrophotoluminescence

In the previous chapter, it was noted that the behavior of luminescence is affected by factors which alter the membrane potential. Although the rate of decay of d.l. was invariant to the salt composition of the reaction medium, the intensity was strongly dependent upon ionic strength, particularly changes in ionic strength induced by the addition of divalent cations. A sudden change in ionic strength, such as that caused by the rapid addition of salt, results in a transitory increase in luminescence intensity [44,91]. The basis for this effect was ascribed to the development of a diffusion potential across the membrane. The rate-limiting step in the build-up of this potential is the intrinsic mixing time of several milliseconds associated with the addition of the stock salt solution to the chloroplast reaction medium. As the membrane potential equilibrated to the sudden change in the surrounding ionic atmosphere, the intensity of the signal decayed to a level slightly below the level which would be observed in the absence of salt additions. This quenching was ascribed to a

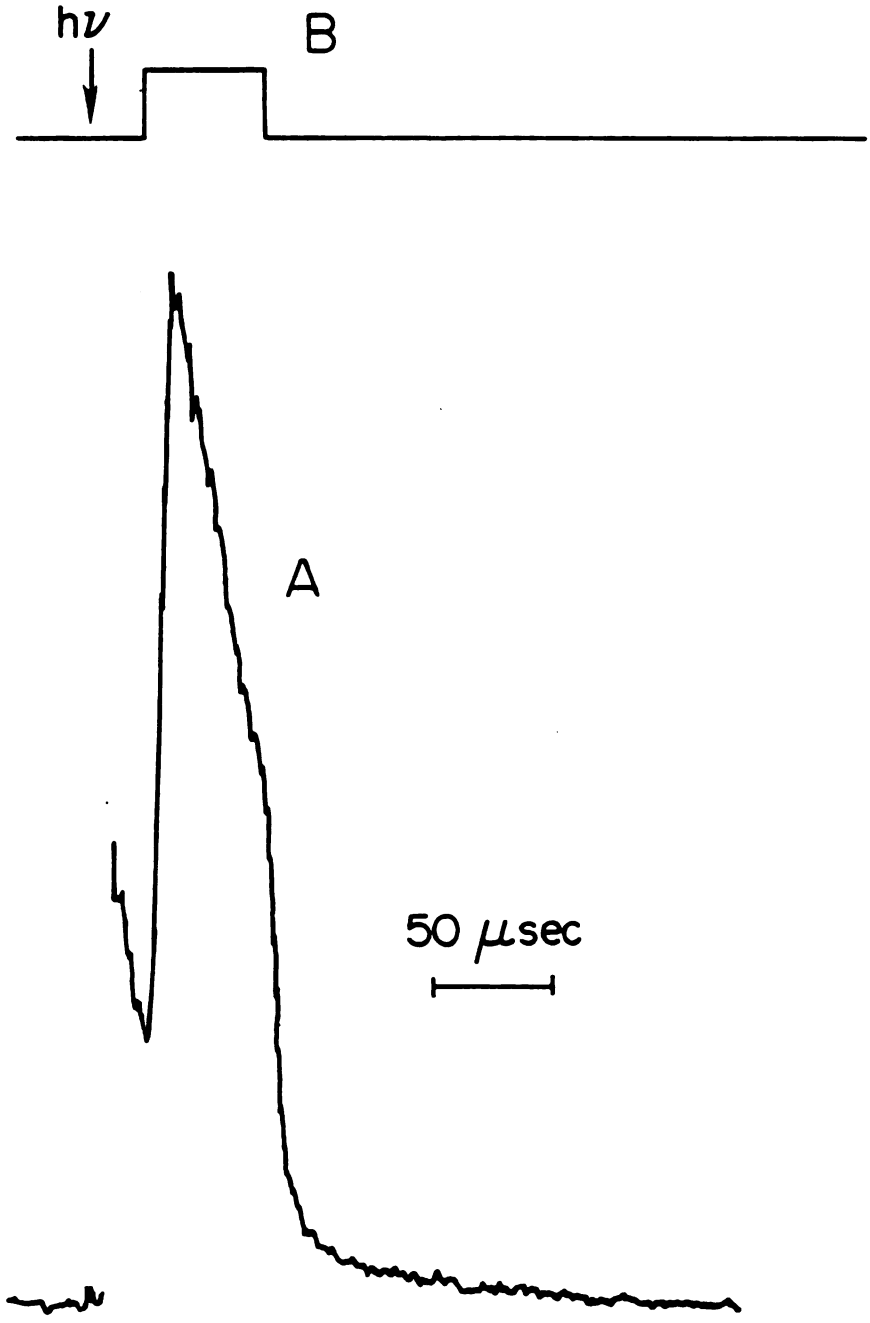
salt induced depletion of luminescence precursors [44,91]. Similar enhancement of luminescence may be induced through the application of an external electric field (see Figure IV-1). The enhanced luminescence is referred to as electrophotoluminescence (EPL) [1,2]. In an EPL experiment, an external electric field is applied at a predetermined dark time (t_d) following illumination of the thylakoids. Upon onset of the field, there is a rapid increase in the intensity of d.l. Upon removal of the field, the d.l. intensity dissipates to the level, within experimental noise, which would be observed had no perturbation been applied. The electric field technique has numerous advantages over the salt-injection method. In a salt-injection experiment, the effect is limited by the mixing time for the salt. Thus, the perturbation is essentially applied after the light induced electron transfer reactions of PSII, which occur in the submillisecond time range, are completed and PSII may be considered to be in a psuedo-equilibrium state. In contrast, the external electric field pulse may be applied within microseconds of laser illumination, hence generating a perturbation on PSII while it is in a transitory state. The risetime for the EPL effect is limited primarily by the resistance of the reaction medium and the capacitance of the thylakoid membrane [92]. Finally, since the perturbation may be removed, recovery effects may be investigated.

FIGURE IV-1

Effect of an externally applied electric field on delayed luminescence in untreated chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) (trace A). The field strength was 1800 V/cm and was applied 20 µs following the third saturating actinic flash. Following the third actinic light flash, a fresh sample was flowed into the sample cell. Shown above the experimental trace is the field profile (trace B). The experimental trace is the average of 7 measurements.

THE EPL EFFECT

Untreated chloroplasts
1800 V/cm, t_d = 20 μsec
pH 7.0, Flash 3



A model for the effect of an externally applied electric field on the thylakoid membrane potential is shown in Figure IV-2. Upon application of an external field, an induced field develops across the membrane. Assuming that the conductivity of the internal and external aqueous phases is much greater than that of the membrane, the magnitude of the induced field may be obtained from standard electrodynamic theory. The model invoked is that of a dielectric sphere in an external field and the magnitude of the induced field across the membrane is given by [92,93]:

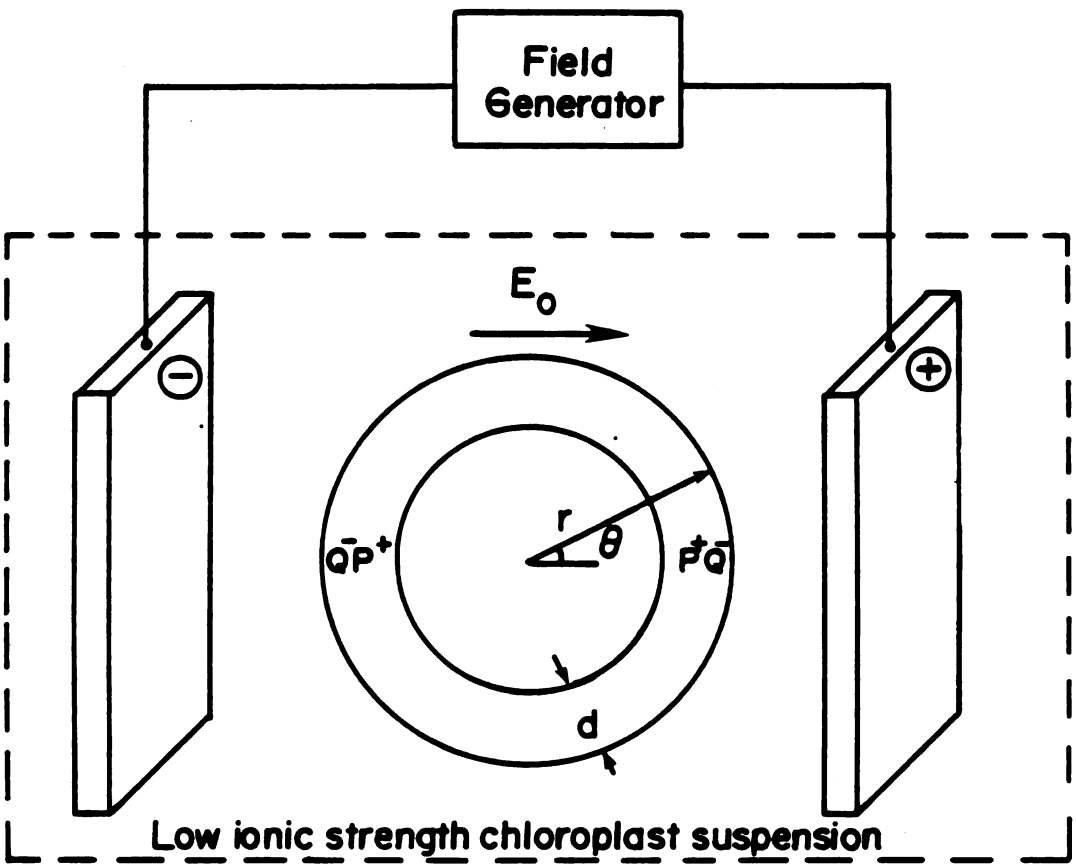
$$E = (3/2)(r/d)\cos(\Theta)E_o \qquad \text{(IV-1)}$$

In Eq. IV-1, E_o refers to the magnitude of the external field, r and d represent the outer radius and thickness of the membrane, respectively, and Θ is the angle between a point on the sphere and the vector originating from the center of the sphere in the direction of the external field. The equation reported by Ellenson and Sauer [2] relating the magnitude of an induced field across a dielectric sphere in the presence of an external field becomes equivalent to Equation IV-1 in the limit in which the thickness (d) is much less than the radius (r). Several important points about the EPL effect become apparent from Eq. IV-1. The $\cos(\Theta)$ dependence implies that the magnitude of the perturbation is maximal at the polar points relative to the external electrodes decreasing to 0 at the longitudinal cross section, i.e., the

FIGURE IV-2

Effect of an external electric field on the membrane potential and on the dipole generated by the photo induced charge separations. The arrows indicate the direction and magnitude of the induced field.

Electrophotoluminescence EPL—Schematic



$$E = \frac{3E_0}{2} \frac{r}{d} \cos\theta$$

$r \approx 2000\,nm, d \approx 5nm$

and for $E_0 = 1000\ V/cm$, $\quad E \cong 600\ E_0 \cos\theta$

$$\frac{EPL}{DL} = \cosh\left(\frac{nFV}{RT}\right) - 1$$

direction in which the induced field is normal to the sphere's surface. The z component of the induced field is in the same direction as that of the applied field. Thus the light induced dipole (P^+Q^-) is destabilized in one hemisphere, stabilized in the other. Additionally, the magnitude of the induced field increases linearly with the radius of the sphere, thus explaining the experimental necessity of suspending the thylakoids in a reaction medium having a low osmotic strength. Upon suspension in a hypotonic medium, the thylakoids balloon up and form spherical vesicles called blebs. Although the distribution of bleb radii is dependent upon the specific reaction medium, typical radii of blebs are around 5000 nm [93]. Thus for a 50 nm thick membrane, the maximal value obtained for the induced field is 150 times that of the applied field; a 1000 V/cm external field generates a local field of up to 1.5×10^5 V/cm. Electric fields of this magnitude should have significant effects on the activation energy of the various electron transfer reactions of PSII [94]. Presumably, the effect of destabilization in one hemisphere is greater than that of stabilization in the other. Hence, there is a net enhancement of luminescence intensity. The fact that the reactions of PSII proceed with high quantum efficiency and the net backreaction occurs in only a fraction of the centers argue for the feasibility of a field induced destabilization of photochemical intermediates of PSII.

In the initial report on the EPL phenomenon of class II chloroplasts isolated from green plants, Arnold and Azzi derived Equation IV-2 to explain the EPL effect [1]:

$$EPL/DL = \cosh(nE)-1 \qquad \text{(IV-2)}$$

In deriving Equation IV-2, it was assumed that there existed 2 distinct populations of luminescence precursors, each at the polar edge of the sphere. In Eq. IV-2, DL is the level of nonperturbed luminescence, EPL is the magnitude of the enhancement, and the argument nE is the factor by which the activation energy for radiative recombination is altered by the field. Experimentally, Eq. IV-2 qualitatively describes the EPL phenomenon observed in the ms time range following illumination. This chapter describes the results of EPL experiments performed in the μs to ms time range.

B. Results

1. Electric Field Origin of EPL

EPL arises from the electrical properties of the system as opposed to thermal effects. By utilizing a Wheatstone conductivity bridge, the specific resistance of the reaction media (10 mM HEPES, pH 7.0) was determined to be 3000 ohm cm, which for a potential gradient of 2000 V/cm generates a current density of 0.66 A/cm^2. The Joule heating would be 1300 J cm^{-3} s^{-1}. Thus for a field applied for 50 μs across 0.25 ml of an aqueous sample (specific heat

= 1 cal K^{-1} g^{-1}), the net increase in temperature would be less than 0.07°C. Additionally, treatments which affect membrane integrity, such as the ionophore gramicidin, tend to eliminate EPL in the ms time range [2]. Similarly, gramicidin eliminates EPL when the perturbation is applied within the μs time range following illumination.

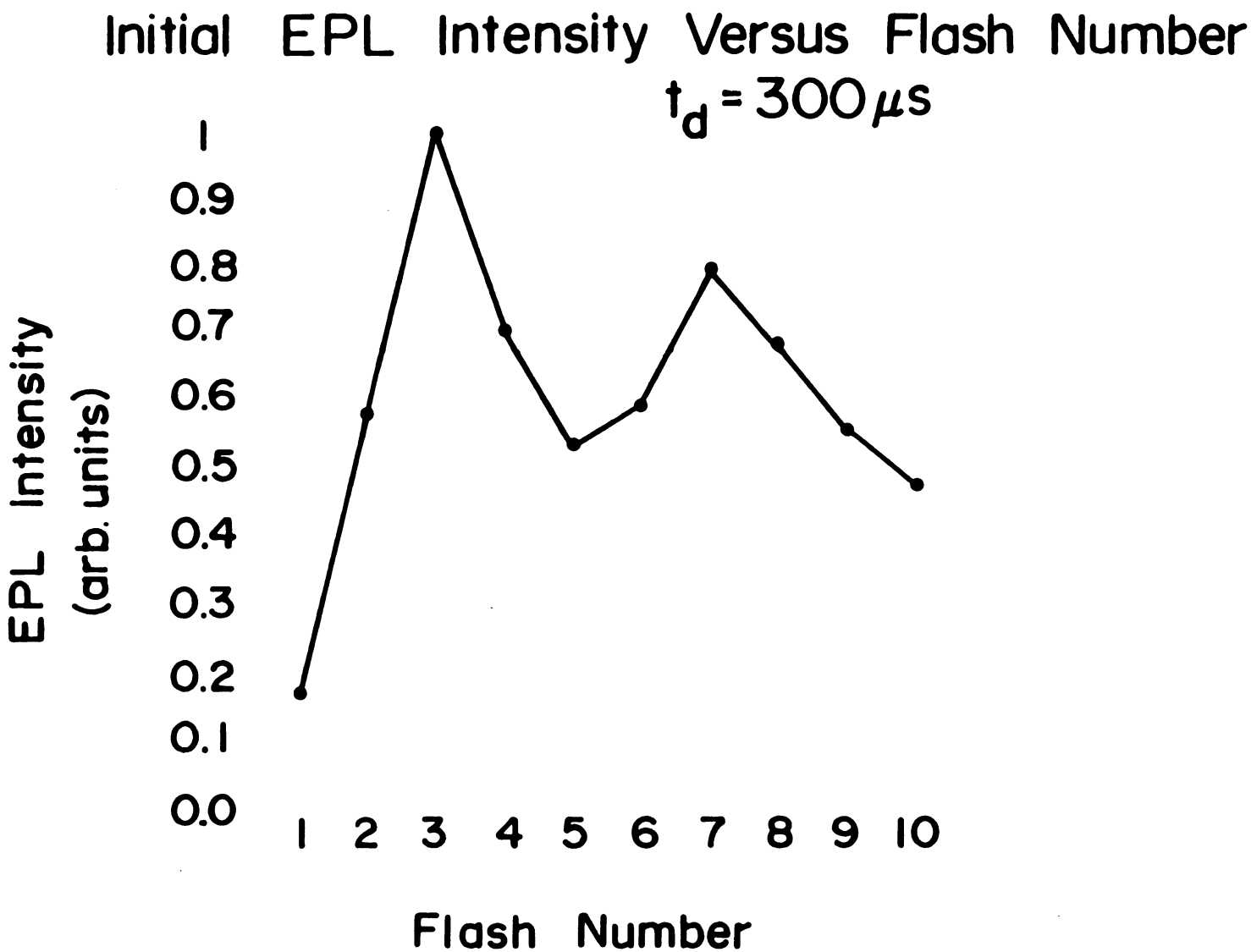
The general behavior of EPL is extremely sensitive to experimental conditions. In general, the EPL intensity induced by a short electric field pulse increases with the age of the stock solution for about thirty minutes; subsequently the intensity remained at a constant level until the sample was about 4 hours old. Because of this time dependence, the stock solution of chloroplasts was replaced after 3 hours to avoid the effects of sample degradation. Upon dilution into the reaction mixture and subsequent 5 minute dark incubation period, the EPL intensity was invariant to further effects of the hypotonic shock treatment for at least 20 minutes. All experiments were performed in this time period.

2. Flash Oscillations of EPL Intensity——Origin in PSII

EPL, like d.l., displays a damped four flash oscillatory behavior [93,95]. This is shown in Fig. IV-3 for a 1000 V/cm electric field pulse applied 300 μs after the final actinic flash. This demonstrates that EPL originates from PSII, as opposed to a field induced PSI luminescence. The relative amplitude of the EPL intensity as a function of flash number was found to be

FIGURE IV-3

Flash induced oscillations in EPL intensity observed in untreated chloroplasts. The flash repetition rate was 1 flash/second. The external field (1000 V/cm) was applied 300 μs after the final actinic flash.


Initial EPL Intensity Versus Flash Number
$t_d = 300\,\mu s$
EPL Intensity (arb. units)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1 2 3 4 5 6 7 8 9 10
Flash Number

strongly dependent upon t_d. The oscillations tend to damp out in the ms time range. More extensive data demonstrating this effect are described below. Furthermore, d.l. and EPL have identical light saturation behavior (see Fig. III-5), thus indicating a close relationship between the origin of d.l. and EPL.

3. EPL Kinetic Behavior in Tris-EDTA Washed Chloroplasts

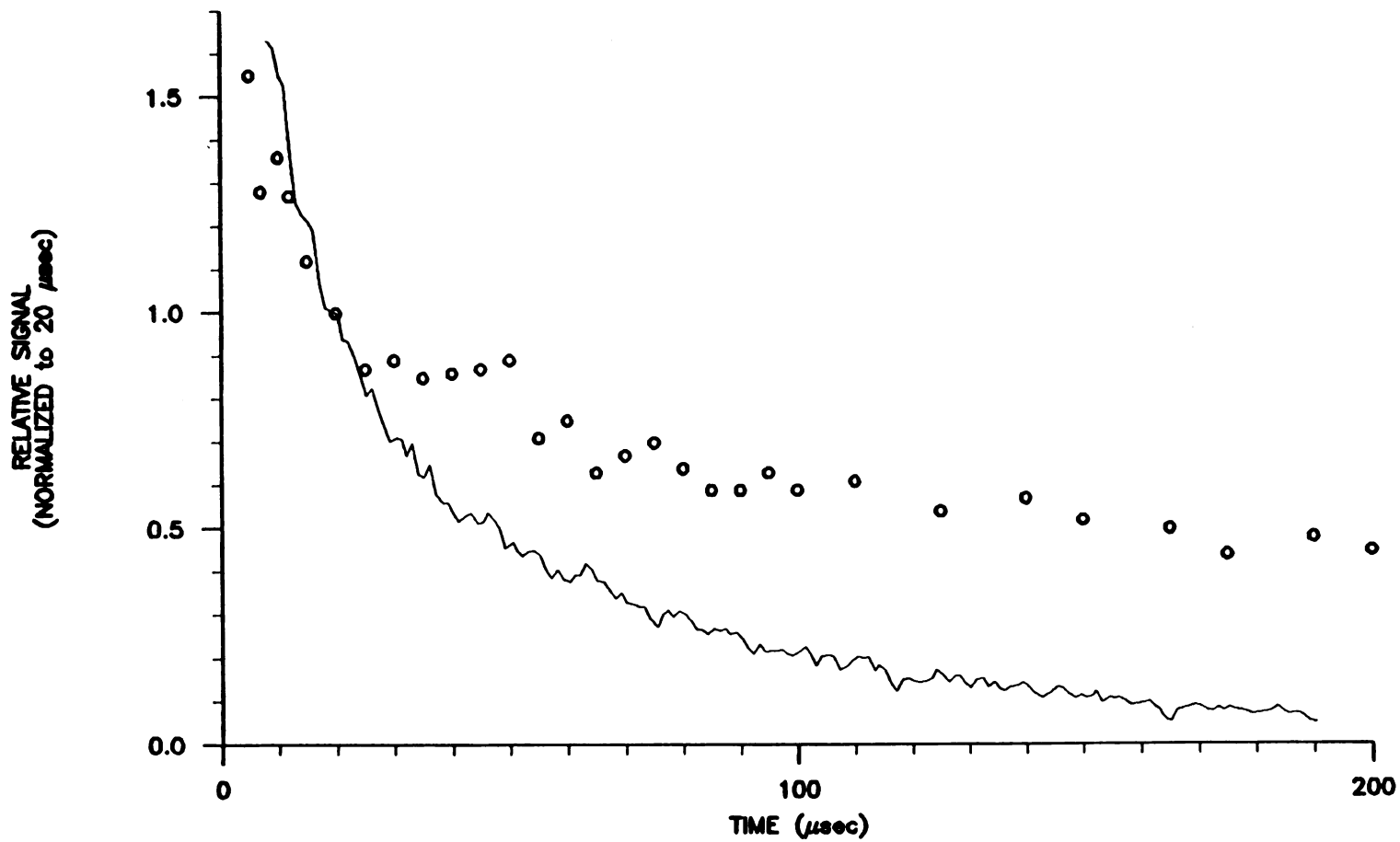
Other aspects of the behavior of EPL, however, differ significantly from that of nonperturbed d.l. The most noticeable effect is on the decay of the EPL intensity which is observed as the field is applied at increasing dark times (t_d) following laser illumination. Kinetic analysis of EPL was usually in terms of the change in maximal induced luminescence as the perturbation is applied at increasing t_d following laser illumination. Figure IV-4 shows normalized decay curves for the maximal EPL intensity analyzed in this manner and d.l. for dark adapted, tris-EDTA washed chloroplasts at pH 7.0. The magnitude of the external field was 1000 V/cm. The most apparent difference between the two curves is the slower decay kinetics associated with EPL. Kinetic analysis of the maximal EPL intensity obtained from t_d in the range of 10 μs to 100 ms indicate that about 15% of the total extrapolated amplitude arises from a component having a halflife of 30 μs. The decay kinetics for EPL also possess phases with halflives of about 8.4 and 110 μs with

FIGURE IV-4

Comparison of the maximal EPL intensity induced by a 1000 V/cm electric field and d.l. at increasing dark times (t_d) following a single light flash in dark adapted tris-EDTA washed chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0).

D.L. & EPL KINETICS

Tris–EDTA washed chloroplasts
pH 7.0, Flash 1



extrapolated amplitudes of 0.5 and 0.35 respectively. The EPL kinetic decay with the 30 ms phases subtracted out is shown in Fig. IV-5. Under these conditions, the initial phase of EPL seems to follow that of d.l., but the intermediate EPL kinetics do not overlap those observed for d.l.

The pH dependence of EPL in dark adapted tris-EDTA washed chloroplasts is shown in Fig. IV-6. Unlike d.l., which displayed a significant pH dependence, the EPL decay curves show an apparent insensitivity to pH in the range of 5.0 to 8.0. For pH 5.0 and 8.0, EPL data were obtained only to 1000 μs; resolution of a 30 ms phase was not possible. Therefore, kinetic analysis was in terms of a biphasic decay for submillisecond EPL, with the resulting pH independent apparent halflives of 20 and 2000 μs. These decay times are probably a convolution of at least three phases ($t_{\frac{1}{2}}$ = 10 μs, 110 μs and 30 ms). The observed pH independence could be a manifestation of the significant contribution of a slowly decaying component on μs EPL which would obscure small differences in the rate of decay, coupled with the experimental difficulty of the EPL technique to resolve slightly different kinetic decay times.

There are thus at least three phases associated with EPL decay in tris-EDTA washed chloroplasts. The first phase ($t_{\frac{1}{2}}$ = 10 μs) is probably, as it is with d.l., a measure of the reduction of $P680^+$ by the endogenous donor, Z. The

FIGURE IV-5

Same conditions as Figure IV-4 except that the 30 ms phase with a relative amplitude of 0.15 is subtacted out of the EPL decay.

D.L. & EPL KINETICS

Tris–EDTA washed chloroplasts
pH 7.0, Flash 1

(The EPL signal is corrected for the 30 msec phase)

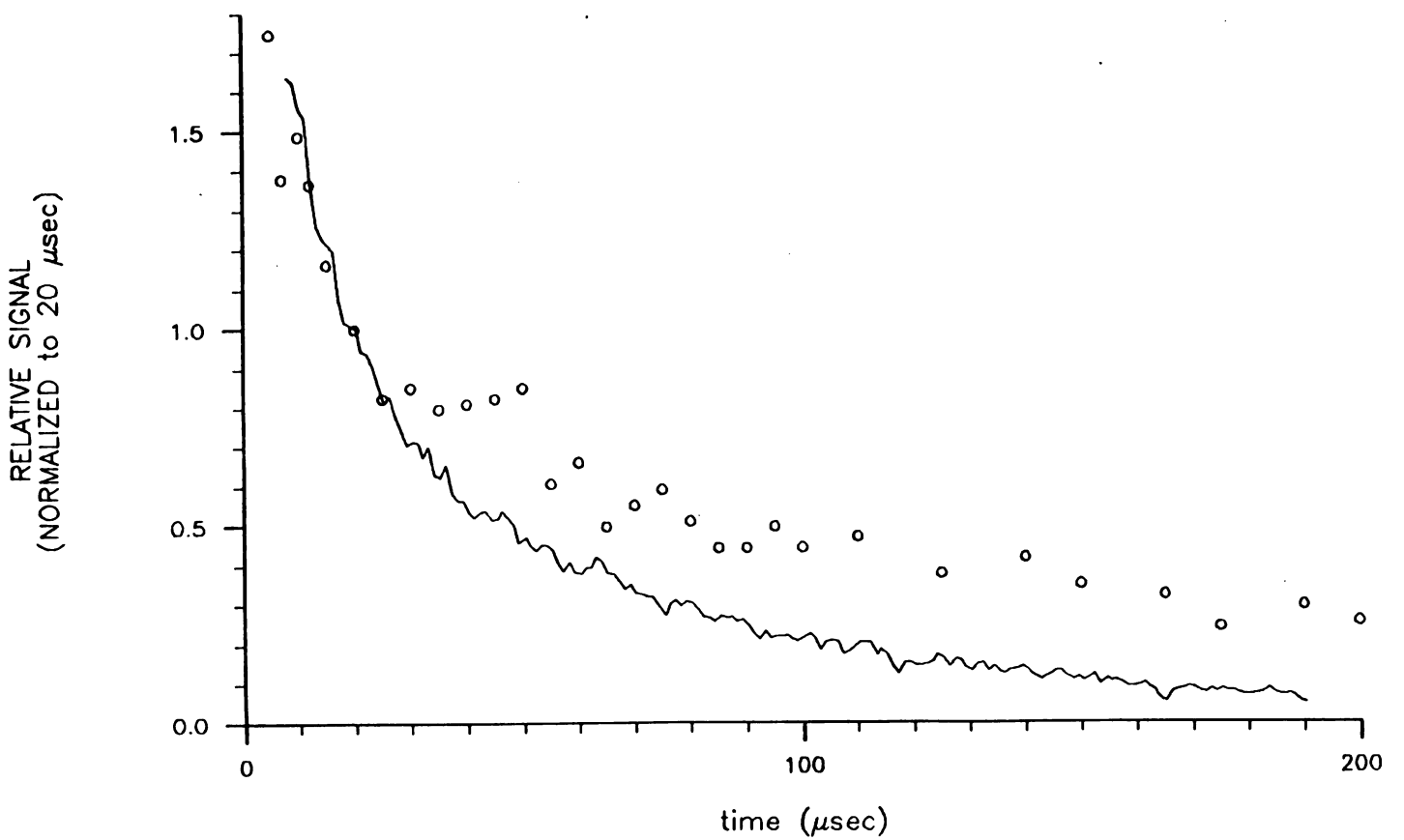
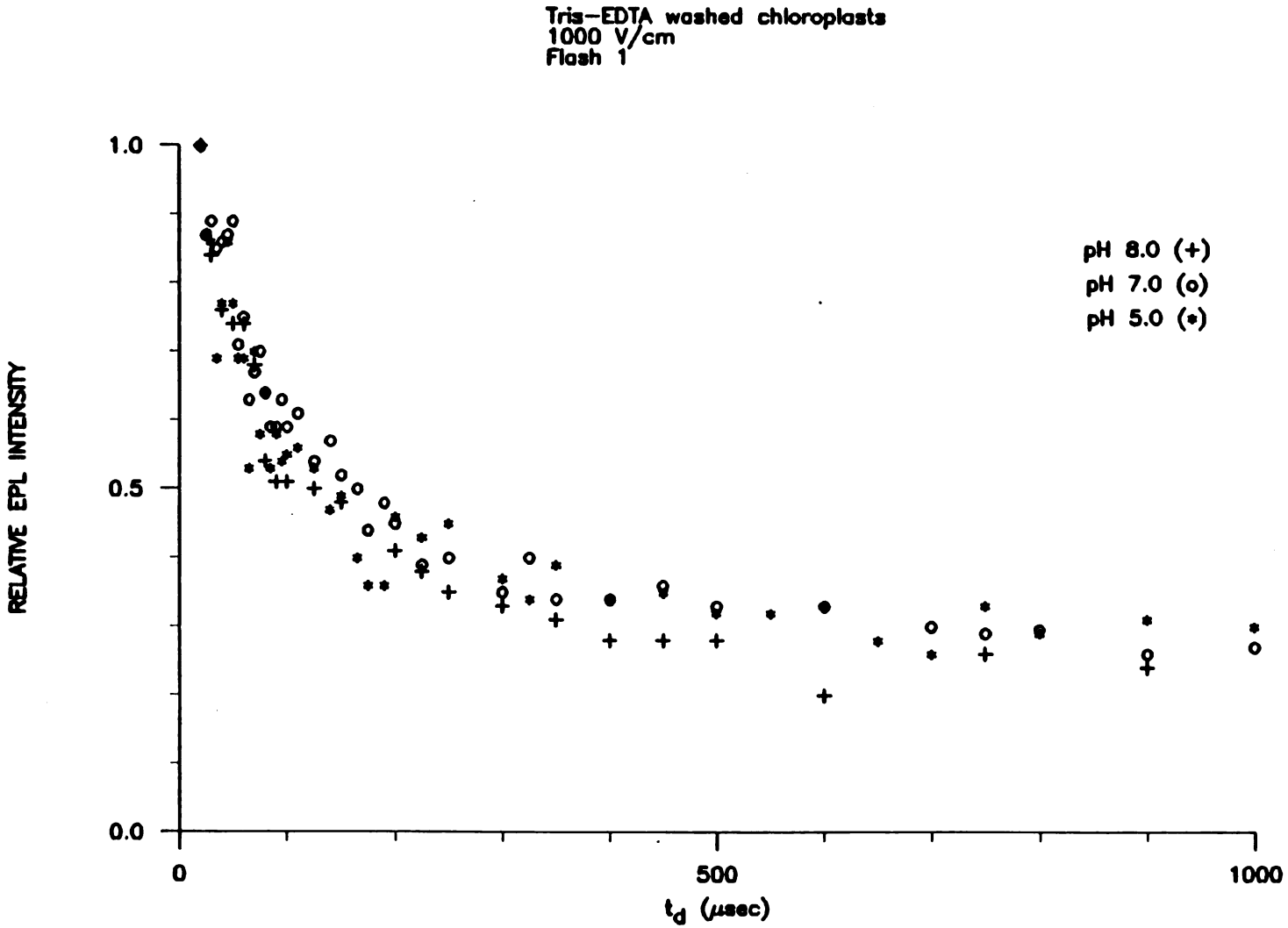


FIGURE IV-6

pH dependence of the EPL decay kinetics in dark adapted tris-EDTA washed chloroplasts (5 µg/ml) suspended in either 10 mM MES (pH 5.0) or 10 mM HEPES (pH 7.0, 8.0) pH 5.0 (*), pH 7.0 (o), and pH 8.0 (+).

RELATIVE EPL INTENSITY vs. t_d



second phase is not so easily understood. Its kinetics and relative amplitude differ significantly from that observed for d.l.; however, it is similar to the pH independent phase reported on $P680^+$ decay by Mathis and coworkers [31]. 100 to 200 μs phases are usually associated with the $P680^+PQ_A^-$ backreaction. The difference in amplitude of the slower phases of EPL relative to d.l. offers some promise for EPL as a probe to investigate the relative normal separation of the light induced dipole at various times after flash illumination. The greater relative intensity of the slower phase may arise from the dipole exposed to a larger fraction of the perturbation (see Figure IV-7).

The 30 ms phase is most likely a measure of the stability of Z^+. This model is substantiated by the effects of exogenous donors on EPL decay kinetics (Fig. IV-8). In Fig. IV-8, the effect of 0.15 mM and 0.30 mM phenylenediamine on EPL decay is shown. The major effect of this donor is on the EPL observed at longer times with little or no effect on EPL in the 10 to 500 μs time range. The long time effect of phenylenediamine can be described by a single exponential decay. The second order rate constant calculated from the pseudo-first order EPL decay at various phenylenediamine concentrations (4.04×10^5 M^{-1} s^{-1} and 4.81×10^5 M^{-1} s^{-1} for 0.15 and 0.30 mM Pd respectively) was nearly identical to that reported for the phenylenediamine mediated reduction of Z^+

FIGURE IV-7

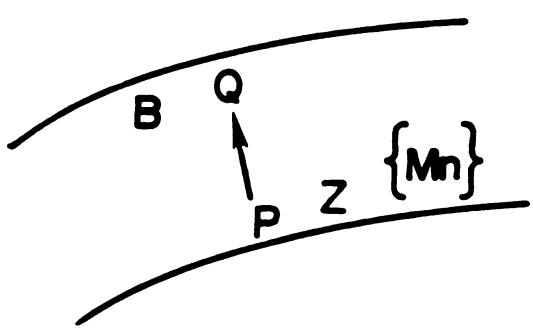
EPL behavior as predicted from Equation IV-2 for two extreme orientations of PSII.

$$\frac{EPL}{DL} = \cosh\left(\frac{nFV_m}{RT}\right) - 1$$

$$V_m = \vec{E}_m \cdot \vec{d}$$

Where $\vec{d}$ = distance between $\oplus$ and $\ominus$

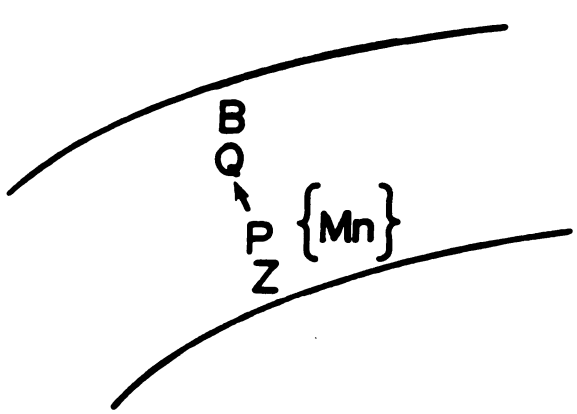
a) "Instantaneous" transmembrane charge separation



$\vec{d}$ constant

$\therefore \frac{EPL}{DL}$ constant

b) Gradual evolution of transmembrane charge separation



$\vec{d}$ increases with time

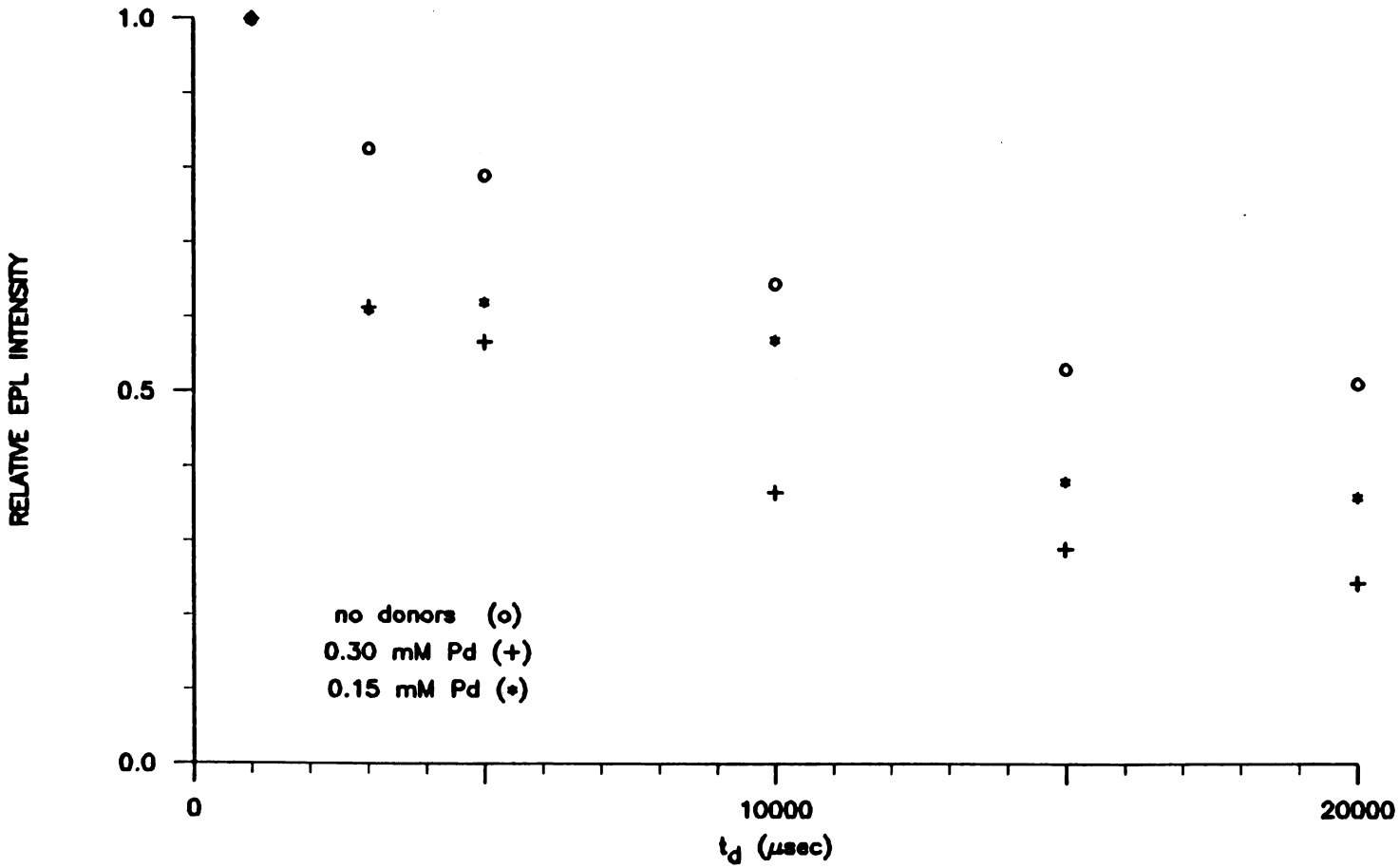
$\therefore \frac{EPL}{DL}$ increases with time

FIGURE IV-8

Effect of various concentrations of phenylenediamine on EPL kinetics in dark adapted tris-EDTA washed chloroplasts (5 µg/ml) in 10 mM HEPES (pH 7.0) plus 0.0 (o), 0.15 mM (*) or 0.30 mM (+)

RELATIVE EPL INTENSITY vs. t_d

Tris–EDTA washed chloroplasts
pH 7, Flash 1
1000 V/cm



as monitored by the ESR Signal IIf (4.5×10^5 M^{-1} s^{-1}) [76]. Alternatively, the addition of Mn^{2+}, an efficient system II donor, increases primarily the rate of decay of EPL in the sub 100 μs time range (see Fig. IV-9). The close agreement between the ESR experiments on Signal IIf [79] indicate that EPL is a sensitive probe to electron transfer reactions of PSII. The enhanced signal/noise ratio and time response of the EPL technique allows for investigations of PSII under conditions which are not easily accessible by ESR such as those events occurring within 100 μs following photochemistry.

4. EPL Kinetic Behavior in Untreated Chloroplasts

The role of Z as a precursor to EPL is further supported by the kinetic behavior of EPL in untreated chloroplasts. The most obvious result is that EPL displays a damped four flash oscillatory behavior as shown in Fig. IV-3. The normalized t_d kinetics for flash number 1 through 3 in untreated chloroplasts is shown in Fig. IV-10. It is apparent that the flash number dependence of EPL is a μs phenomenon; at about 3 ms the EPL intensity is essentially flash number independent. The μs kinetics following flash number 4 was consistently less than that of flash number 3, but for the sake of clarity, the data are not shown in Fig. IV-10. There thus exists a dependency on flash number for the decay kinetics of EPL. This phenomenon corresponds to the well documented dependence of

FIGURE IV-9

Effect of 1.0 mM Mn^{2+} (*) on EPL kinetics in tris-EDTA washed chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0).

RELATIVE EPL INTENSITY vs. t_d

Tris–EDTA washed chloroplasts
pH 7, Flash 1,
1000 V/cm

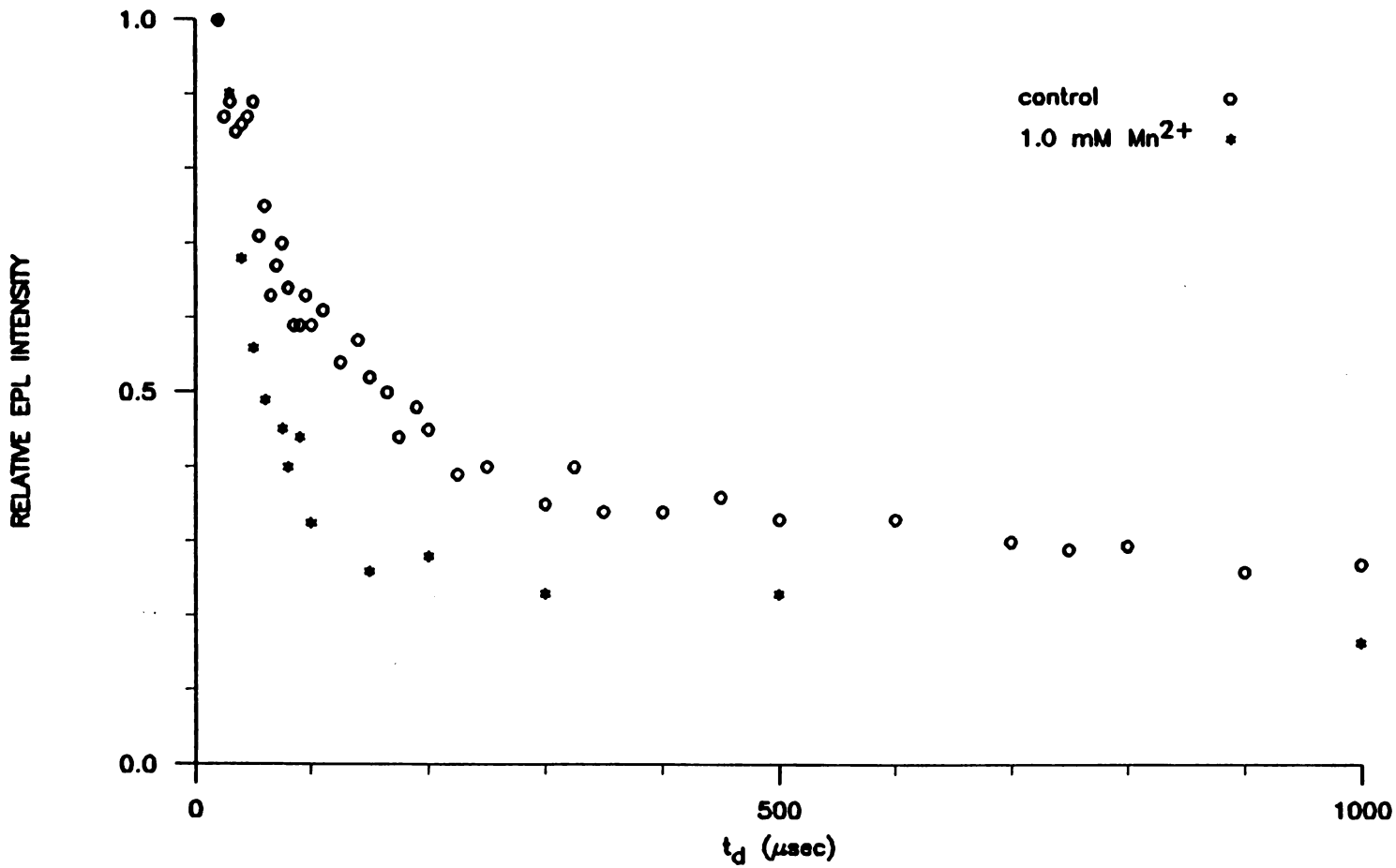
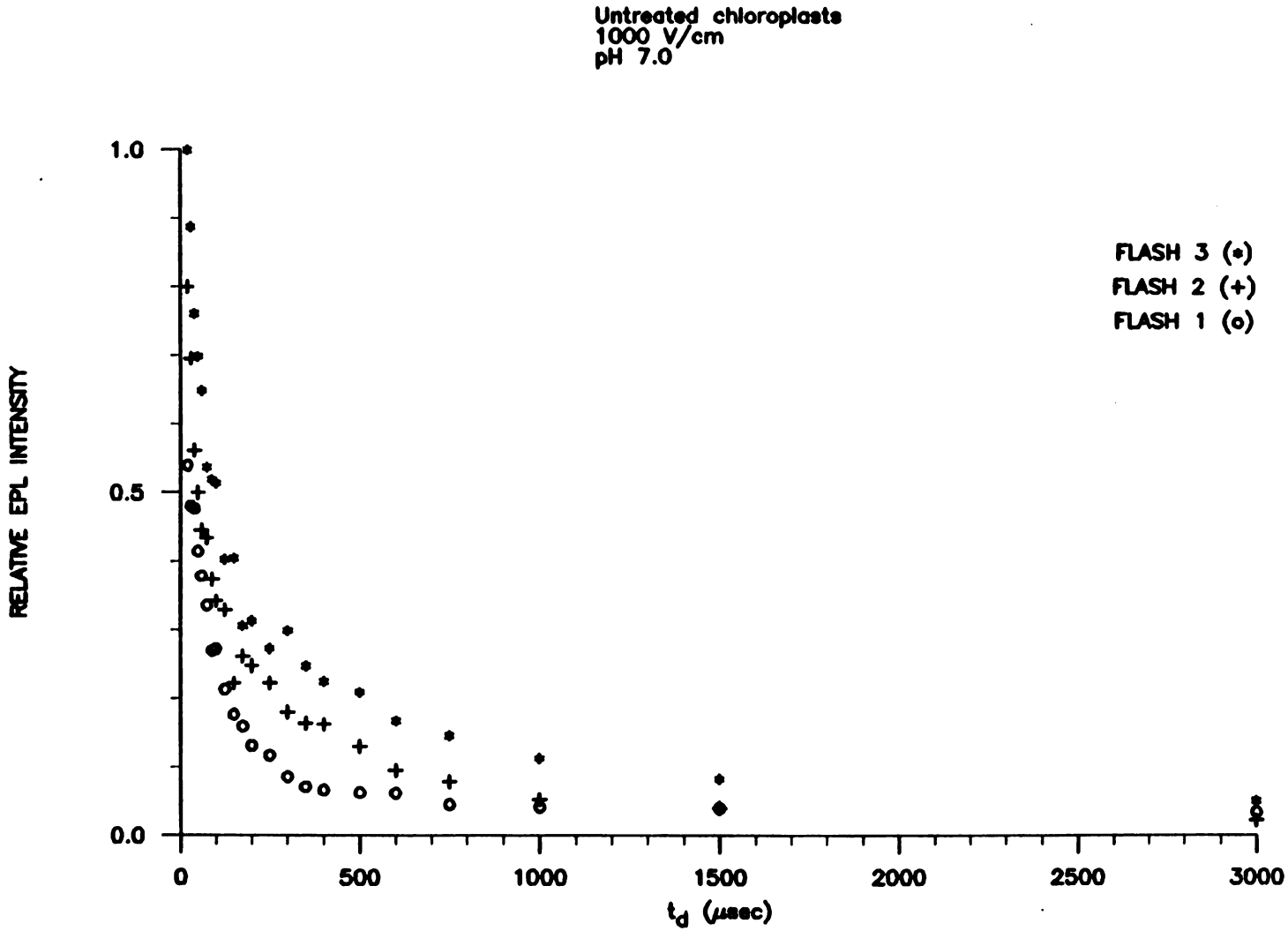


FIGURE IV-10

Flash number dependence of EPL kinetics in untreated chloroplasts (5 μg/ml) suspended in 10 mM HEPES (pH 7.0). Each point was normalized to the EPL intensity observed 20 μs after the third flash. The flash repetition rate was 1 flash/second.

RELATIVE EPL INTENSITY vs. t_d



the Z^+ lifetime on flash number in dark adapted chloroplasts: the rate of Z^+ reduction by the OEC ranges from 100 μs (for S_1 to S_2) to 1 ms (for S_3 to S_4). The EPL decay in untreated chloroplasts follows the accepted decay rate for Z^+ under these conditions. Similarly, PQ_A^- oxidation shows flash oscillations ($t_{½}$ = 200-400 and 600-800 μs following odd and even flashes respectively), and therefore the role of PQ_A on EPL decay must be considered. Electron transport on the reducing side of PSII should be essentially unaffected by tris-washing. In tris-washed chloroplasts, the EPL decay is invariant to flash number between 10 ms and 20 ms (see Fig. IV-11). This indicates that variations in the rate of oxidation of PQ_A^- following flash 1 ($t_{½}$ = 200 to 400 μs) and flash 2 ($t_{½}$ = 600 to 800 μs) do not affect the kinetics of EPL. Thus, as indicated above, EPL is most sensitive to the oxidizing side of PSII.

5. Effect of the Magnitude of the Applied Electric Field on EPL

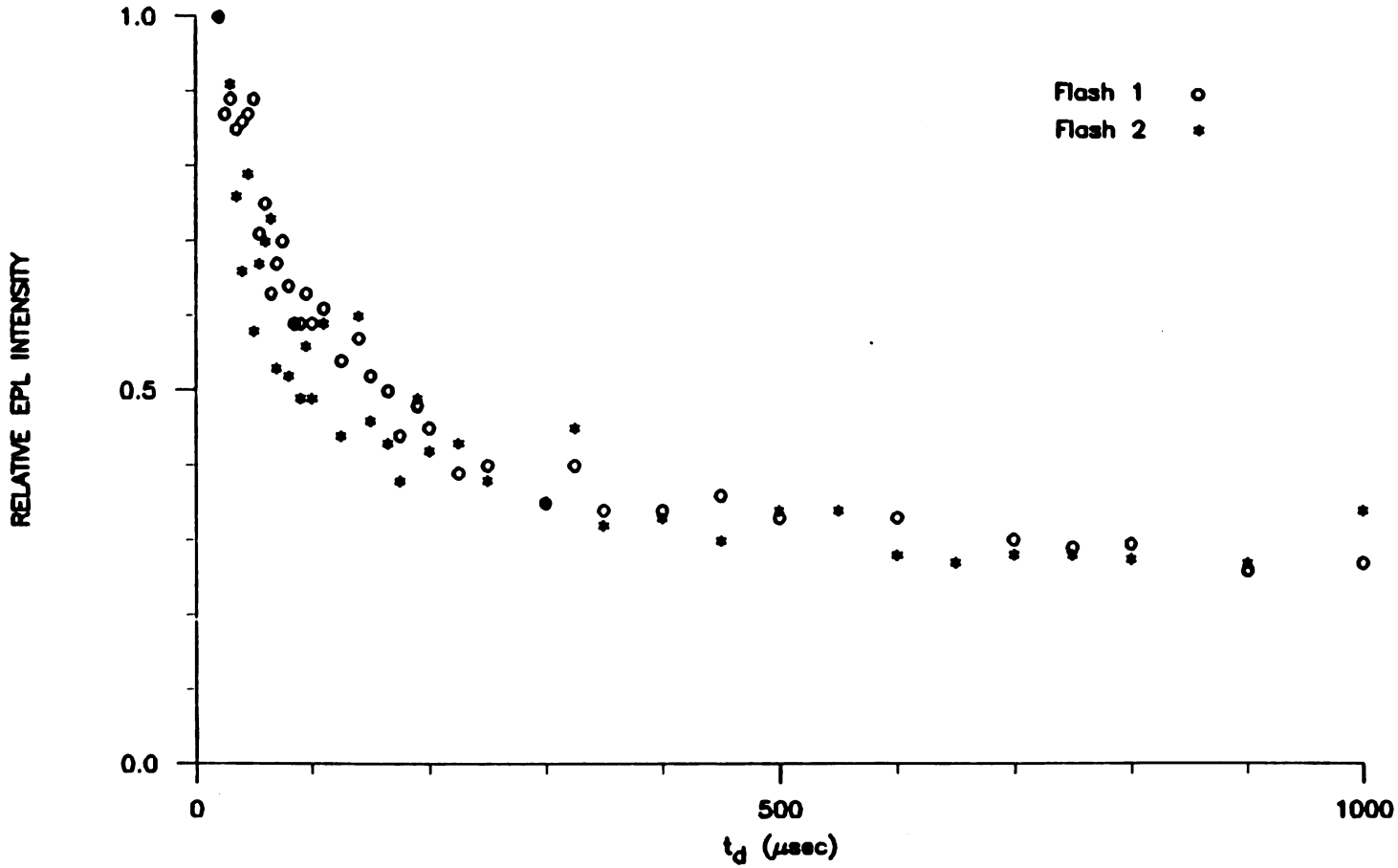
Figure IV-4 depicts a comparison of normalized EPL kinetics to those of the decay of d.1. Even after correcting the EPL signal for the 30 ms decay component, there were major differences in the kinetic behavior. As indicated in the model depicted in Fig. IV-7, the origin of this could be due to a time dependence in the distance separating the charges associated with the light induced dipole. As the electron transfer reactions proceed, specifically

FIGURE IV-11

Flash number dependence of EPL kinetics in tris-EDTA washed chloroplasts (5 μg/ml) suspended in 10 mM HEPES (pH 7.0). Each point was normalized to the EPL intensity observed after the first or second laser flash. The flash repetition rate was 1 flash/second.

RELATIVE EPL INTENSITY vs. t_d

Tris–EDTA washed chloroplasts
1000 V/cm
pH 7.0

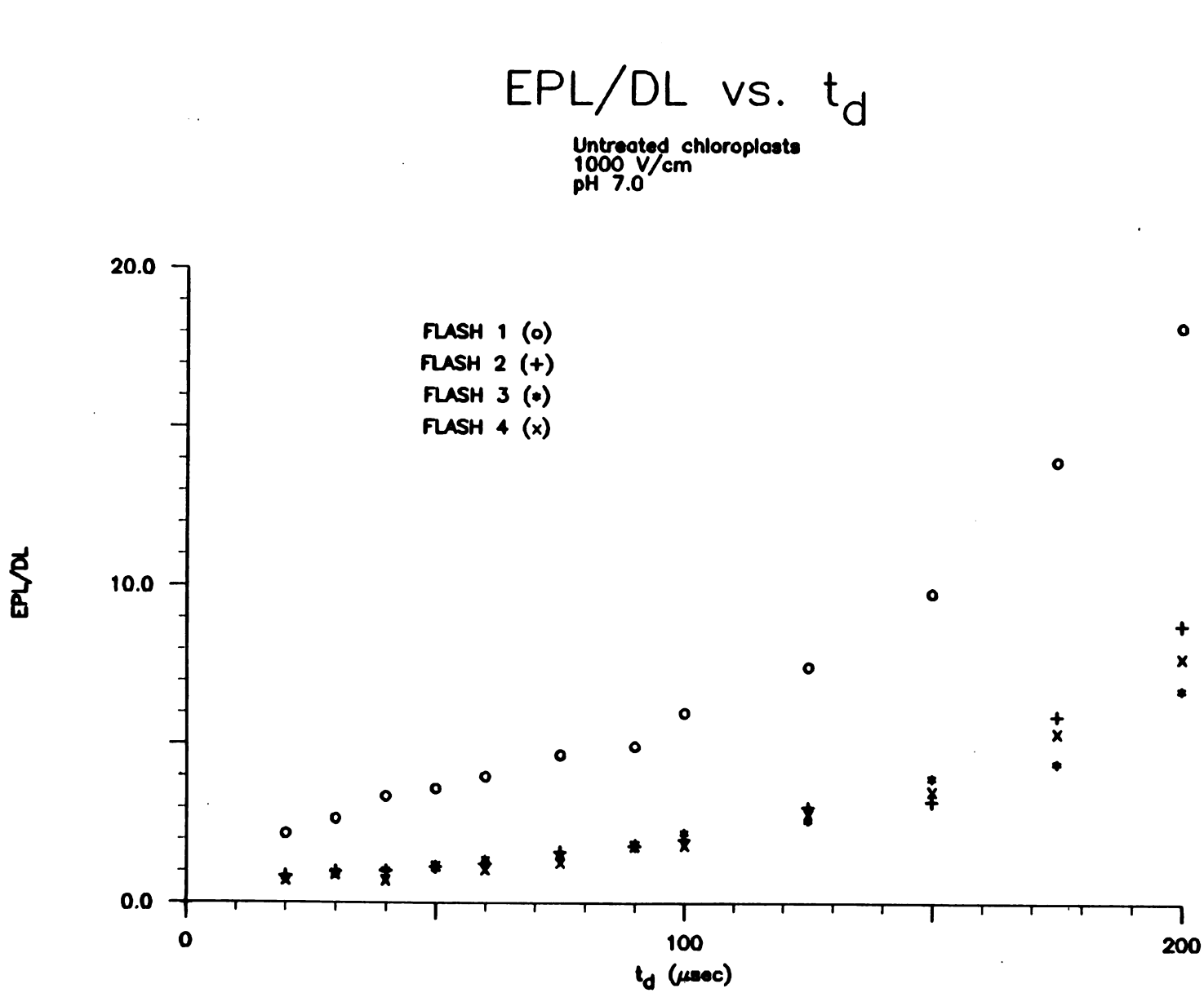


the oxidation of Z and reduction of PQ_A^-, the electron and hole would tend to move away from each other. Thus the distance between and relative orientation of the recombining charge should change in time. The fraction of the perturbation which is felt by the dipole should be proportional to the projection of the dipole on the induced electric field vector. Thus the EPL intensity observed at short t_d times should display a different behavior when compared to the EPL intensity observed at intermediate to long t_d times if electron transfer resulting in charge separation across the membrane occurs in distinct sequential steps. This time dependence of the enhancement (EPL/d.l.) may indicate that the charges tend to move across the membrane in a sequential stepwise manner. Thus at longer times, the light induced dipole (Z^+PQ^-) is being destabilized to a greater extent than at short times (ZP^+Q^-). Plots of EPL/d.l. show a marked time dependence (see Fig. IV-12) which explains the previously reported observation that the enhancement of millisecond d.l. intensity is by a factor of about 100 [2,92] whereas when an external electric field is applied at 20 μs, the EPL intensity is only about 3 times the intensity observed for d.l.

The halflife of $P680^+$ reduction in tris-washed chloroplasts at pH 5.2 is about 17 μs. Thus it is quite possible to apply an electric field when PSII is primarily in the P^+Q^- state. In contrast, at about 100 μs, PSII should be in the Z^+PQ^- state, and at 1 ms the state Z^+PQB^-

FIGURE IV-12

Plot of EPL/d.l. for untreated chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) in the 20 to 200 µs time range. The flash repetition rate was 1 flash per second.


EPL/DL vs. t_d
Untreated chloroplasts
1000 V/cm
pH 7.0
FLASH 1 (o)
FLASH 2 (+)
FLASH 3 (*)
FLASH 4 (x)
EPL/DL
20.0
10.0
0.0
0
100
200
t_d (μsec)

should dominate. (Note that B refers to the secondary quinone acceptor.) If the external field is inducing a luminescence charge recombination, the effectiveness of the field should be related to the relative orientation of the precursors. Figure IV-13 shows a plot of normalized EPL intensity as a function of field strength for t_d times ranging from 15 to 1000 μs in dark adapted tris-EDTA washed chloroplasts suspended in a pH 5.2 reaction medium. The colinear curves indicate that there is an insensitivity to t_d for the effect of field strength on the magnitude of EPL. Similarly, under conditions in which Z is oxidized by a preillumination flash, the relative EPL field dependence which is observed following a second flash is invariant to t_d (Fig. IV-14). A comparison of Figures IV-14 and IV-15 shows that the relative EPL field dependences following flashes 1 and 2 were identical. Similar results were obtained for tris-washed chloroplasts at pH 7.0 (Fig. IV-15). The improved resolution was due to an increase in EPL intensity at pH 7.0. The increase in EPL intensity is perhaps related to the size of the blebs. Because of changes in electrostatic attraction, chloroplasts suspended in a low pH medium may contract which would result in a smaller effective perturbation (see Eq. IV-1). Untreated chloroplasts display a similar insensitivity to field strength. Figures IV-16, IV-17, IV-18 show plots of EPL intensity versus field strength for increasing dark times after 1, 2, and 3, preilluminating flashes respectively.

FIGURE IV-13

Electric field dependence on the relative EPL intensity for dark adapted tris-EDTA washed chloroplasts (5 µg/ml) suspended in 10 mM MES (pH 5.2) for t_d ranging from 15 to 1000 µs following illumination.

RELATIVE EPL INTENSTIY vs. E_{ext}

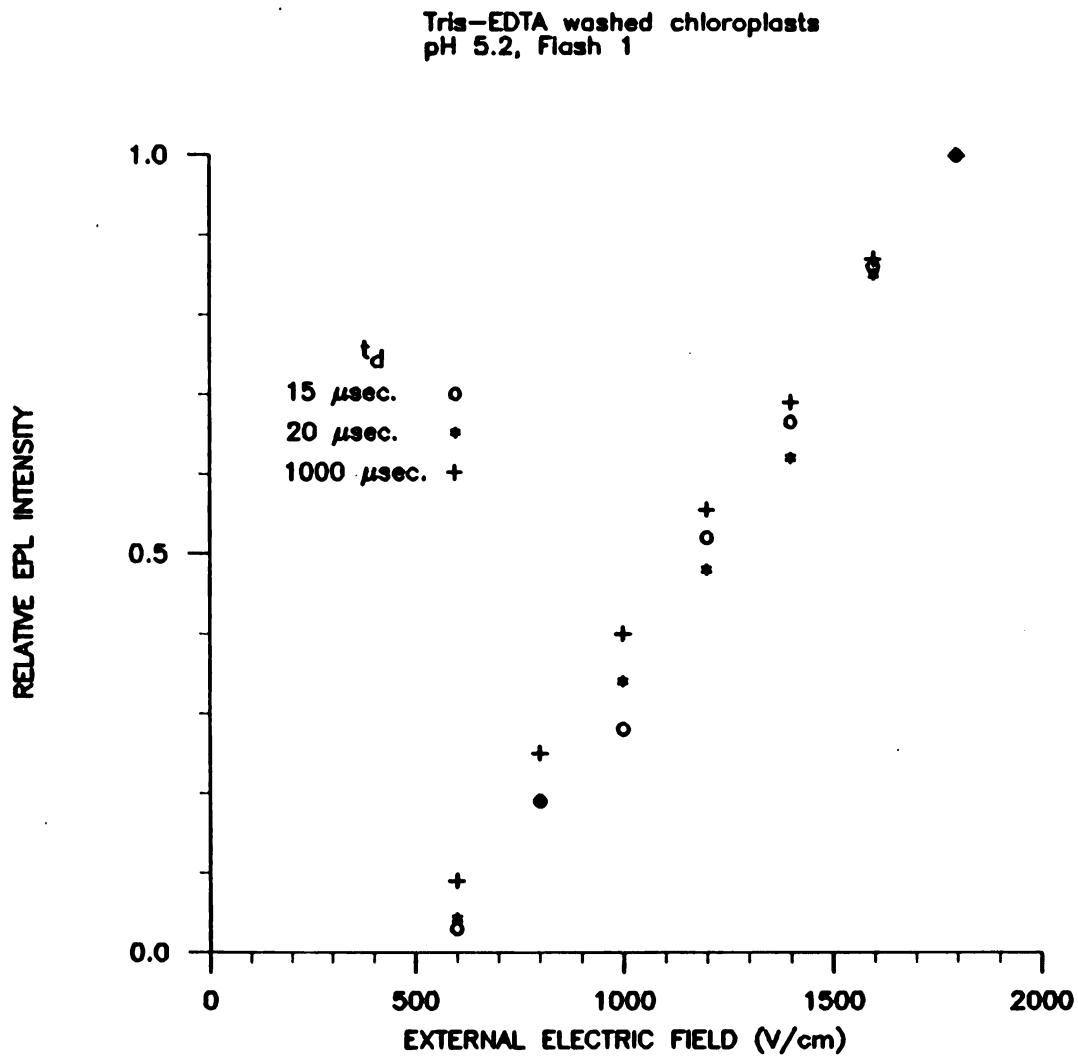


FIGURE IV-14

Electric field dependence on the relative EPL intensity following the second actinic flash in dark adapted tris-EDTA washed chloroplasts (5 µg/ml) suspended in 10 mM MES (pH 5.2) for t_d ranging from 20 to 1000 µs after illumination. The flash repetition rate was 1 flash/second.

RELATIVE EPL INTENSTIY vs. E_{ext}

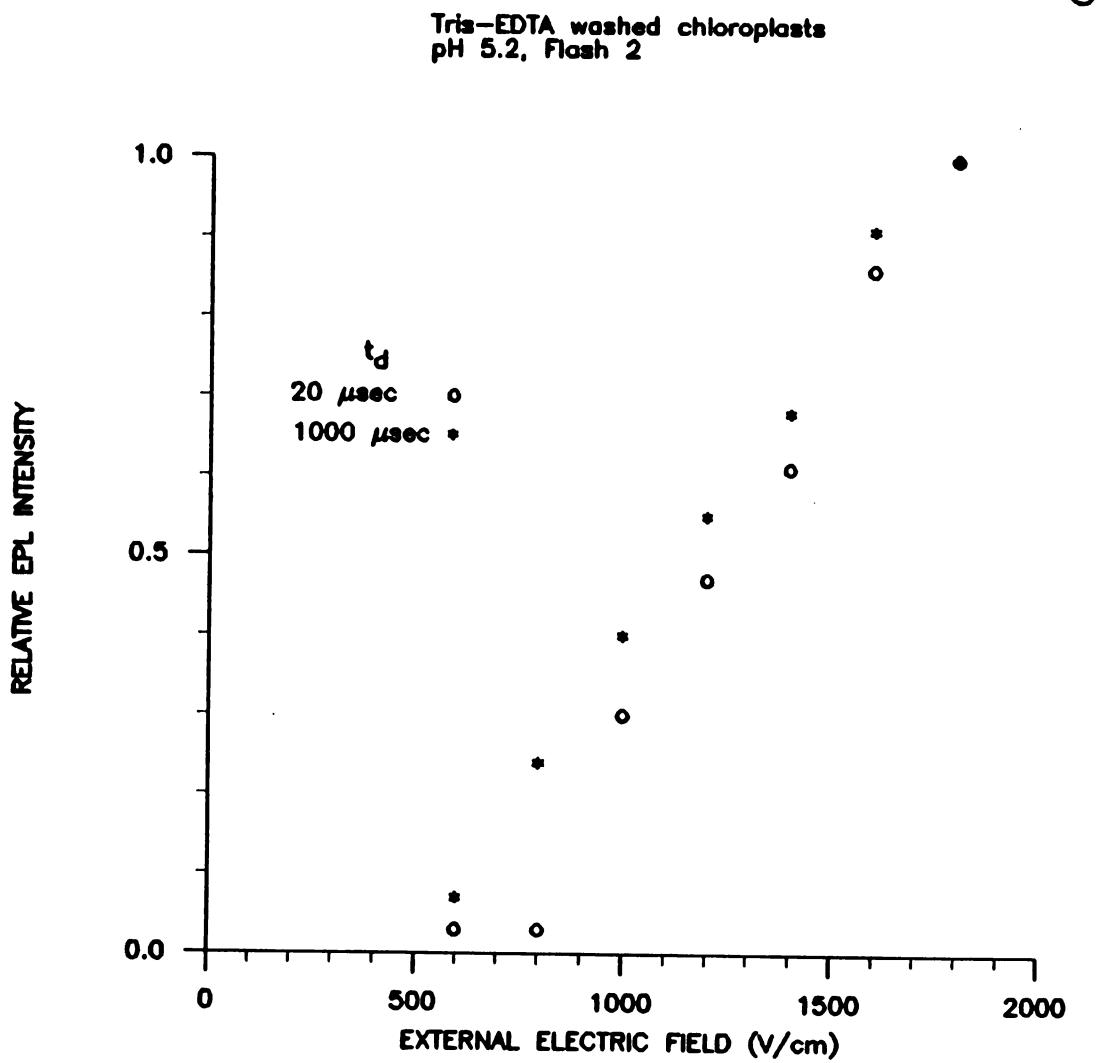


FIGURE IV-15

Electric field dependence on the relative EPL intensity for dark adapted tris-EDTA washed chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) for t_d ranging from 20 to 1000 µs following illumination.

RELATIVE EPL INTENSTIY vs. E_{ext}

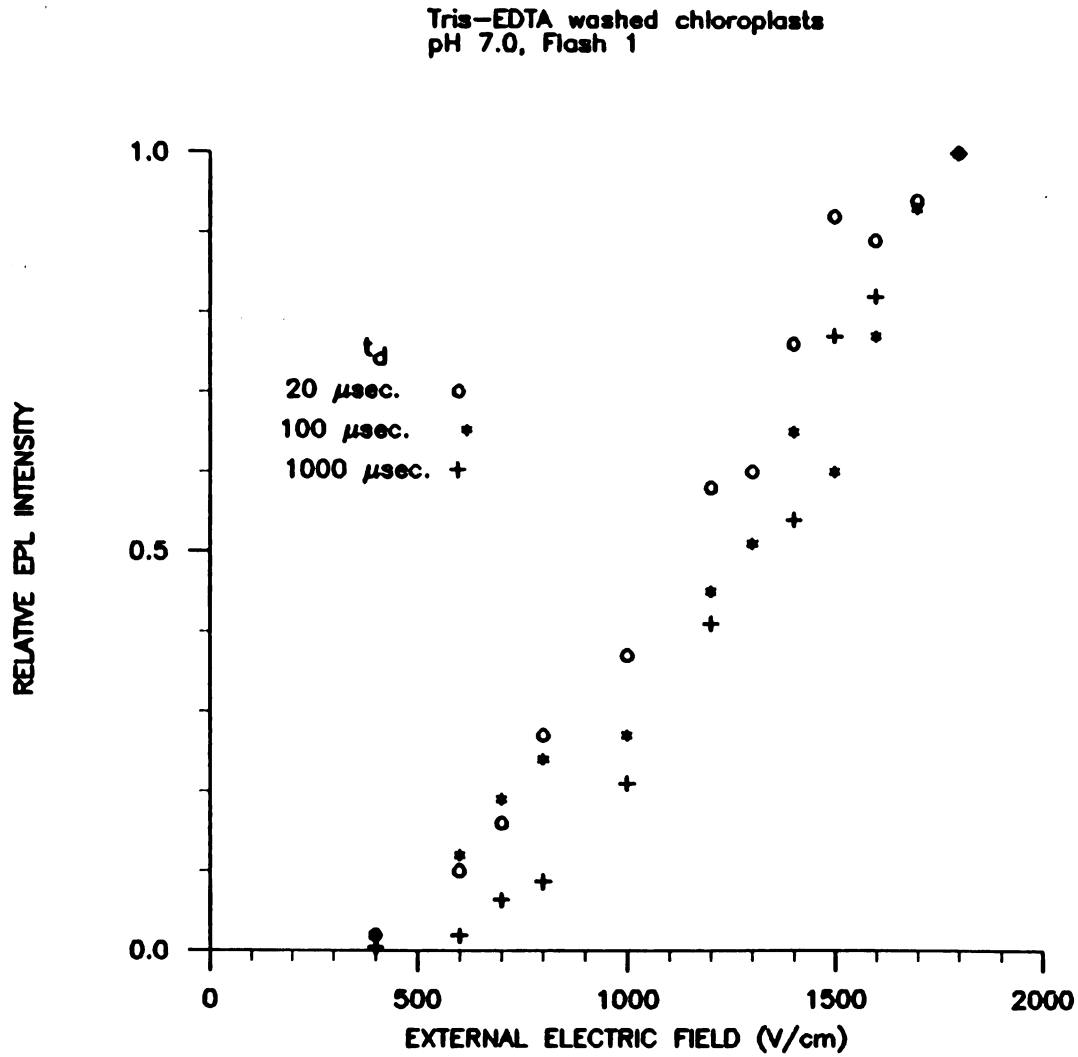


FIGURE IV-16

Electric field dependence on the relative EPL intensity for untreated dark adapted chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) for t_d ranging from 20 to 3000 µs following illumination.

RELATIVE EPL INTENSTIY vs. E_{ext}

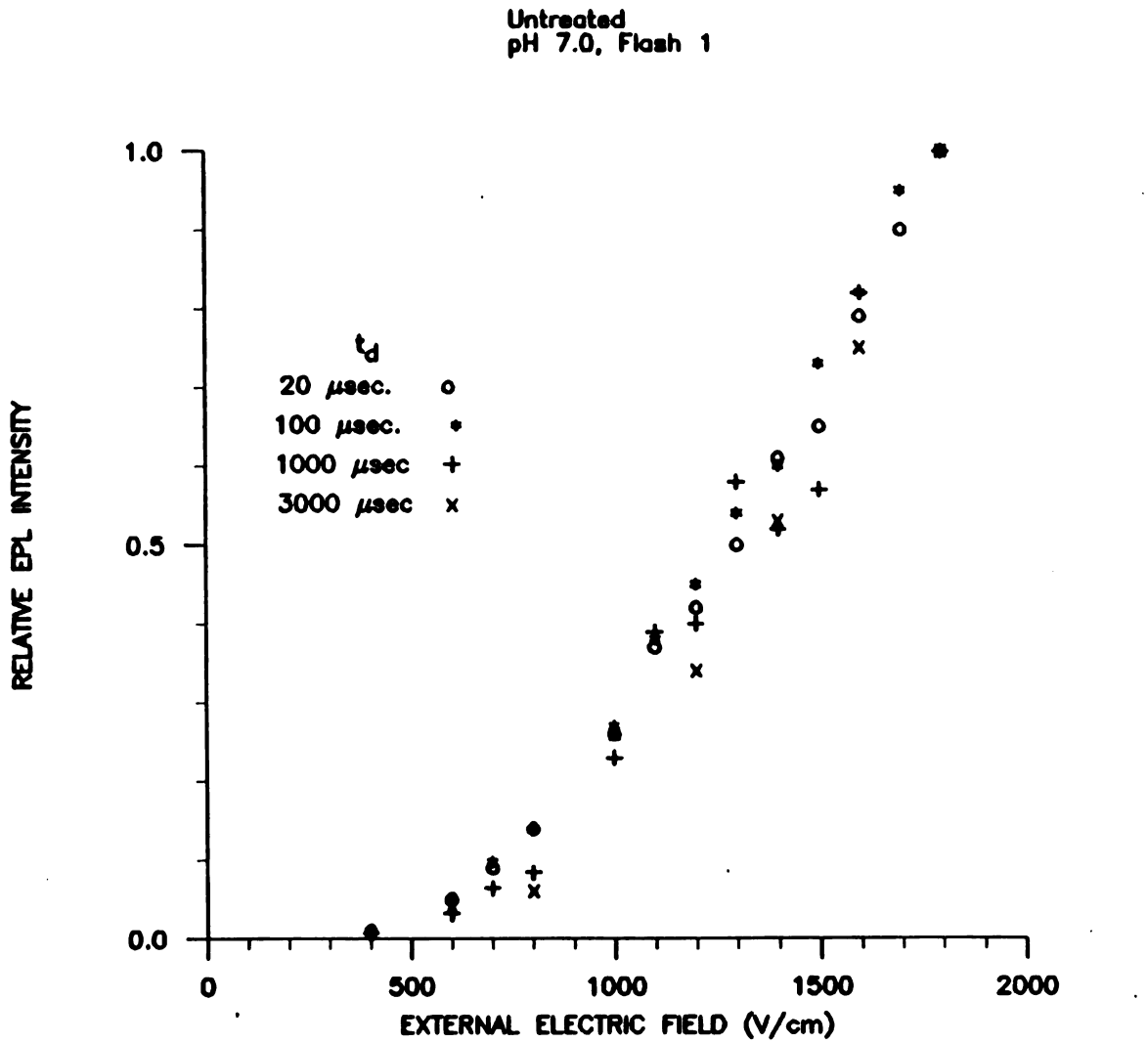


FIGURE IV-17

Electric field dependence on the relative EPL intensity for untreated dark adapted chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) following flash number 2 for t_d ranging from 20 to 1000 µs. The flash repetition rate was 1 flash/second.

RELATIVE EPL INTENSTIY vs. E_{ext}

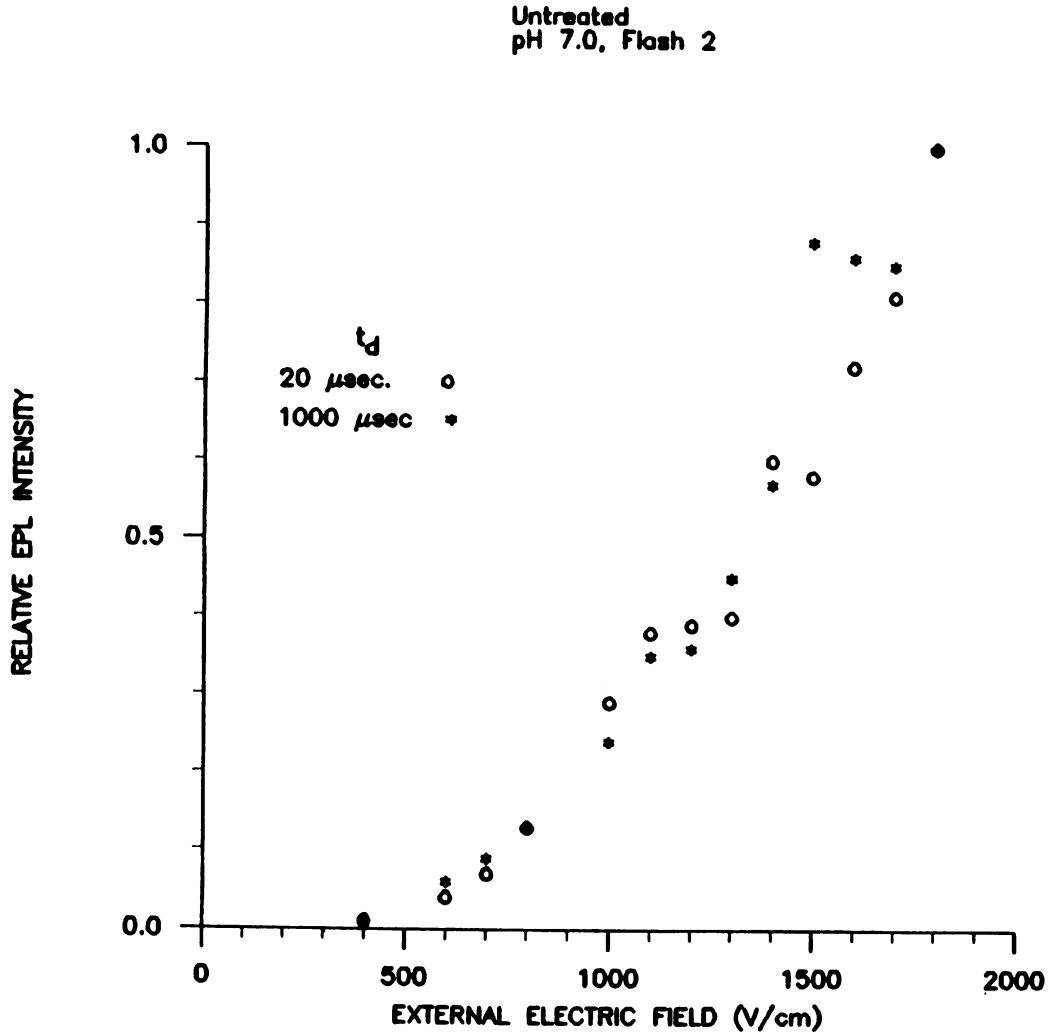
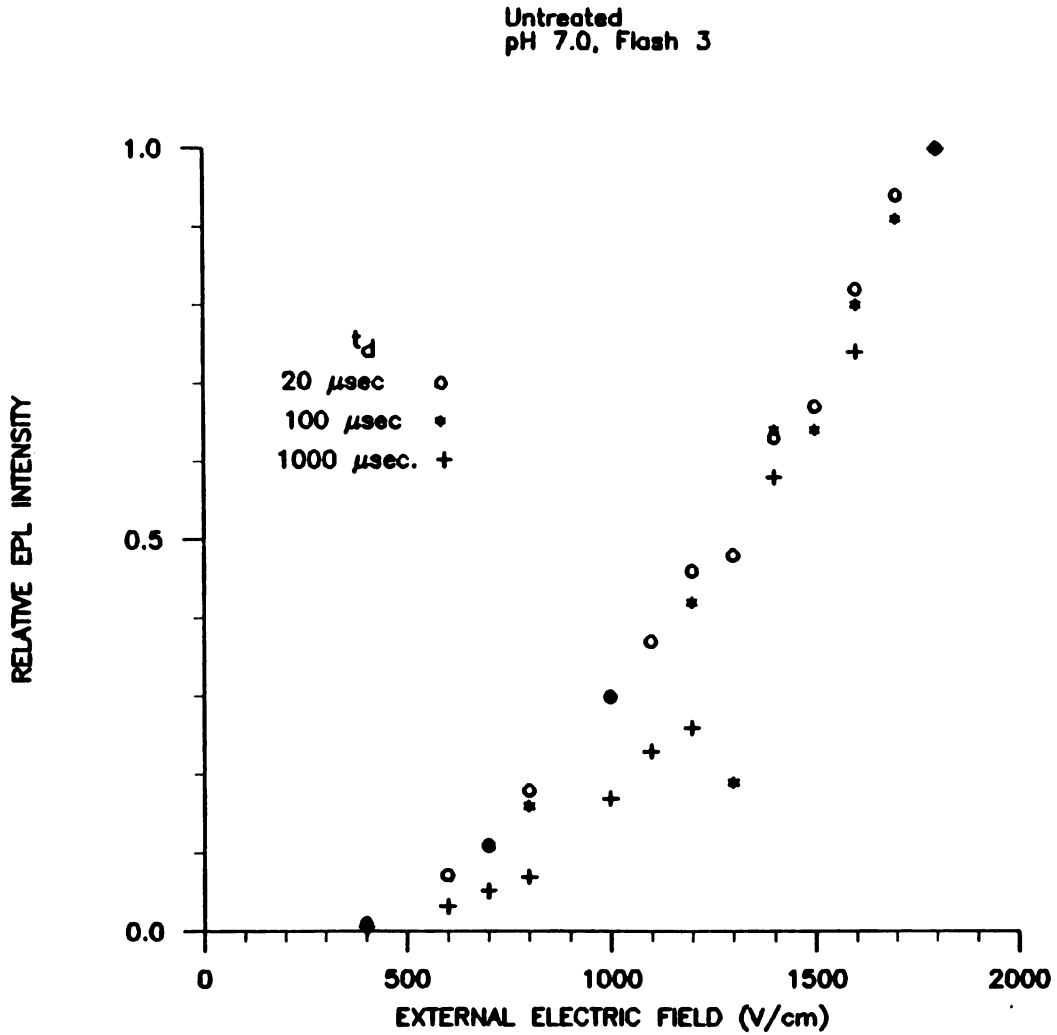


FIGURE IV-18

Electric field dependence on the relative EPL intensity for untreated dark adapted chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) following flash number 3 for t_d ranging from 20 to 1000 µs. The repetition rate was 1 flash/second.

RELATIVE EPL INTENSTIY vs. E_{ext}



This is summarized for flash numbers 1 and 3 by the insensitivity of EPL decay kinetics to the magnitude of the perturbation (Figures IV-19 and IV-20). Regardless of pretreatment, dark time following illumination, and flash number, the dependence of the induced luminescence as a function of the magnitude of the perturbation, were proportional. This indicates either that the transmembrane charge transfer occurs in either a single step and that secondary electron transfer reactions proceed in a tangential manner within the membrane, or that EPL is not sensitive enough to detect a slight t_d dependence in the field strength effect. It is also possible that the EPL arises from effects other than that of an induced backreaction; for example, the exciton or luminescence yield may be increased. We have not explored the possible effects of these latter two phenomena in any detail.

FIGURE IV-19

t_d plot for dark adapted chloroplasts (5 µg/ml) suspended in 10 mM HEPES (pH 7.0) for external electric fields of 1000 (o) or 1800 (+) V/cm.

RELATIVE EPL INTENSITY vs. t_d

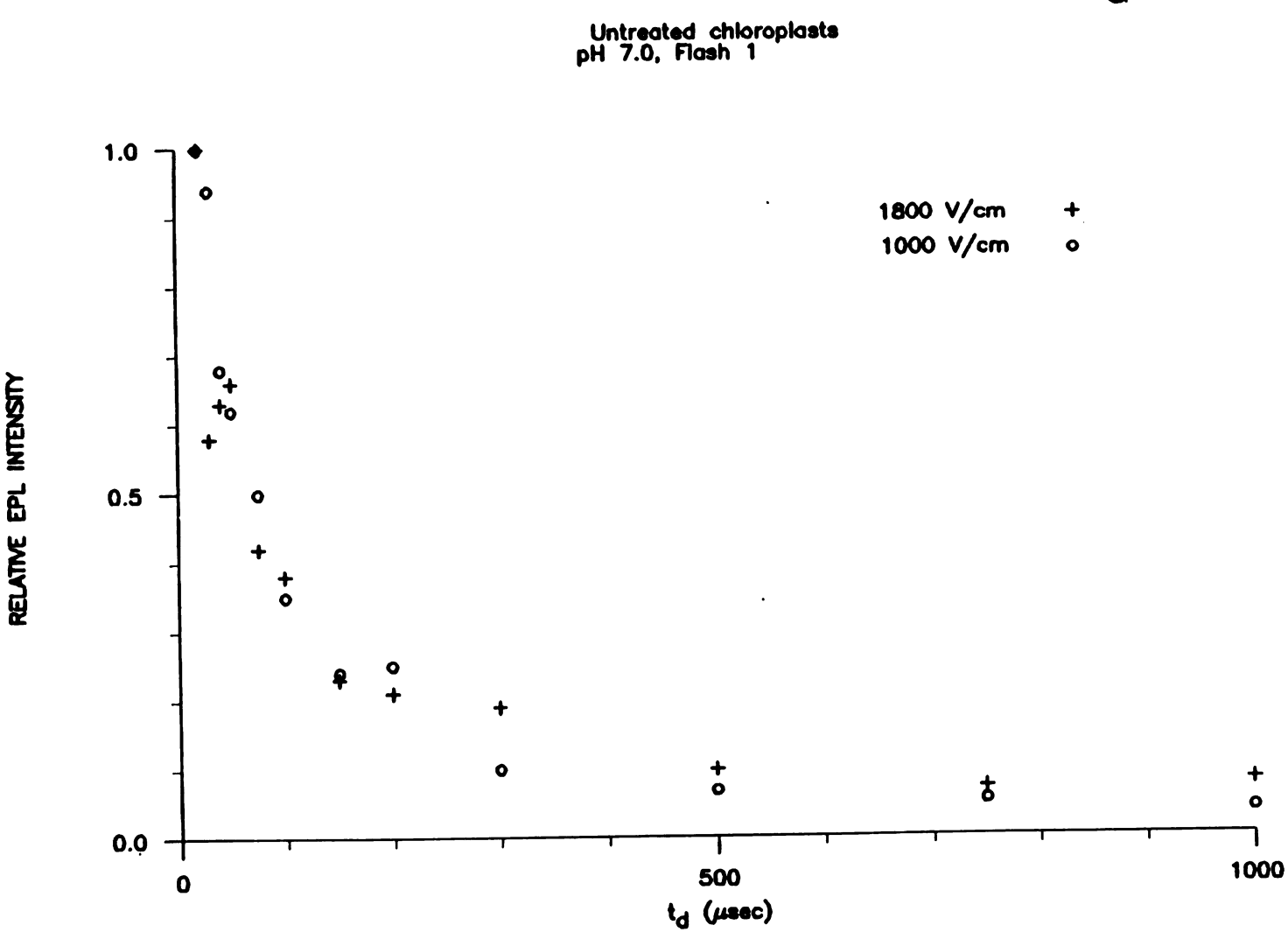
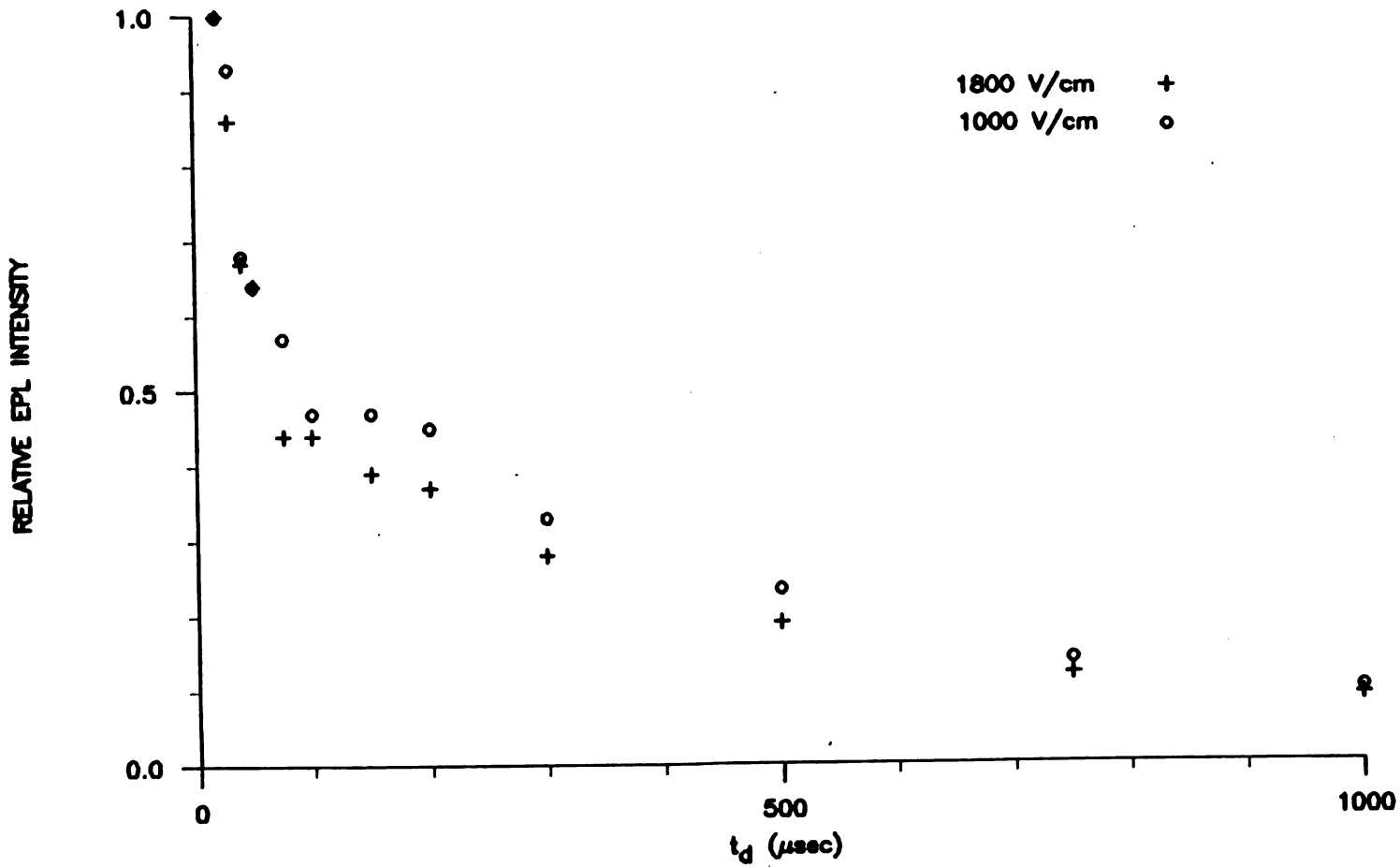


FIGURE IV-20

Same conditions as Figure IV-19 except that the EPL was induced by flash number 3.

RELATIVE EPL INTENSITY vs. t_d



CHAPTER 5

MODEL STUDIES

SIMPLE ELECTRON TRANSFER MODEL FOR DELAYED LUMINESCENCE

In chloroplasts which have electron transport inhibited between P680 and the OEC, such as for tris-washed chloroplasts, the electron transport scheme in dark adapted samples following a single turnover may be represented as

$$\begin{array}{ccccc} ZPQB & \xleftarrow{k_b} & ZP^+QB^- & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & Z^+PQB^- \\ & & \Big\uparrow \Big\downarrow k_3 \quad k_{-3} & & \Big\uparrow \Big\downarrow k_2 \quad k_{-2} \\ & & ZP^+QB^- & \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} & Z^+PQB^- \end{array} \qquad \text{(V-1)}$$

In Eq. V-1, Q and B represent PQ_A and PQ_B respectively, as defined in Chapter 1 and Fig. I-2, P is the P680-Pheophytin complex and Z is the physiological donor to P680. Reduction of PQ_A or $P680^+$ by the reduced pheophytin proceeds with a halftime of less than 200 ps [16] or 5 ns [67] respectively. Thus for events occurring on the μs time scale, the role of the pheophytin need not be explicitly considered. The complicated scheme depicted by Eq. (V-1) may be easily simplified. The rate of reduction of $P680^+$ is over an order of magnitude faster than PQ_A^- oxidation.

Hence the step associated with the rate constant k_3 may be neglected. Moreover, if only submillisecond events are considered, the backreaction between B and Q^- is negligible. Therefore, within the constraints of these two approximations, the reactions associated with k_4, k_{-4} and k_{-3} may be omitted and submillisecond electron transport for tris-EDTA washed chloroplasts may be represented as:

$$X3 \xleftarrow{k_b} X1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X2 \xrightarrow{k_2} X4 \qquad \text{(V-2)}$$

In Eq.(3.2), X3 is the fully relaxed state (ZPQB), X1, X2, and X4 represent the states ZP^+QB^-, Z^+PQB^- and Z^+PQB^-, respectively. The rate of change of these states is simply:

$$\frac{d[X1]}{dt} = -(k_1 + k_b)[X1] + k_{-1}[X2] \ , \qquad \text{(V-3a)}$$

$$\frac{d[X2]}{dt} = k_1[X1] - (k_{-1} + k_2)[X2] , \qquad \text{(V-3b)}$$

$$\frac{d[X3]}{dt} = k_b[X1] , \qquad \text{(V-3c)}$$

and

$$\frac{d[X4]}{dt} = k_2[X2] , \qquad \text{(V-3d)}$$

The differential equations in (V-3a) and (V-3b) are coupled and may be solved by standard methods for systems of linear, first-order differential equations to obtain explicit expressions for X1 and X2; these expressions may then be used in (V-3c) and (V-3d) to solve for X3 and X4. Invoking

the boundary condition that at time = 0, all centers are in the P^+Q^- (X1) state, a condition which would correspond to fully dark adapted samples illuminated by a single saturating light pulse, the solution to (V-3) is:

$$[X1] = A1_- \exp(L_- t) + A1_+ \exp(L_+ t) \qquad \text{(V-4a)}$$

$$[X2] = A2_- \exp(L_- t) + A2_+ \exp(L_+ t) \qquad \text{(V-4b)}$$

$$[X3] = A3_-\{1 - \exp(L_- t)\} + A3_+\{1 - \exp(L_+ t)\} \qquad \text{(V-4c)}$$

$$[X4] = A4_-\{1 - \exp(L_- t)\} + A4_+\{1 - \exp(L_+ t)\} \qquad \text{(V-4d)}$$

where

$$L_\pm = \frac{-(k_1+k_b+k_{-1}+k_2) \pm R}{2}$$

$$A1_\pm = \frac{\pm(k_{-1}+k_2) \mp (k_1+k_b) + R}{2R}[X1]_o$$

$$A2_\pm = \frac{\pm k_1 [X1]_o}{R}$$

$$A3_\pm = \frac{k_b\{R \pm (k_1+k_b) \mp (k_{-1}+k_2)\}}{R(k_1+k_b+k_{-1}+k_2 \mp R)}[X1]_o$$

$$A4_\pm = \frac{\pm 2k_1k_2 [X1]_o}{R(k_1+k_b+k_{-1}+k_2 \mp R)}$$

with

$$R = [(k_1+k_b-k_{-1}-k_2)^2 + 4k_1k_{-1})]^{\frac{1}{2}}$$

In tris-EDTA washed, DCMU treated chloroplasts, secondary electron transport on the reducing side of PSII is

inhibited; i.e. $k_2 = 0$. Thus the reaction scheme depicted in Eq. (V-2) is simplified to:

$$X3 \xleftrightarrow{k_b} X1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X2 \qquad \text{(V-5)}$$

where X1, X2, X3, k_1, k_{-1}, and k_b are defined as for Eq. (V-2). The only assumption invoked for Eq. (V-5) to describe electron transport in tris, DCMU inhibited chloroplasts is that since reactions involving the pheophytin intermediate are fast relative to k_1, k_{-1}, and k_2, the role of the pheophytin in electron transport need not be explicitly considered. Equation (V-4) is the solution to the differential equations associated with (V-5) under conditions in which $k_2 = 0$ as well as $k_2 \neq 0$.

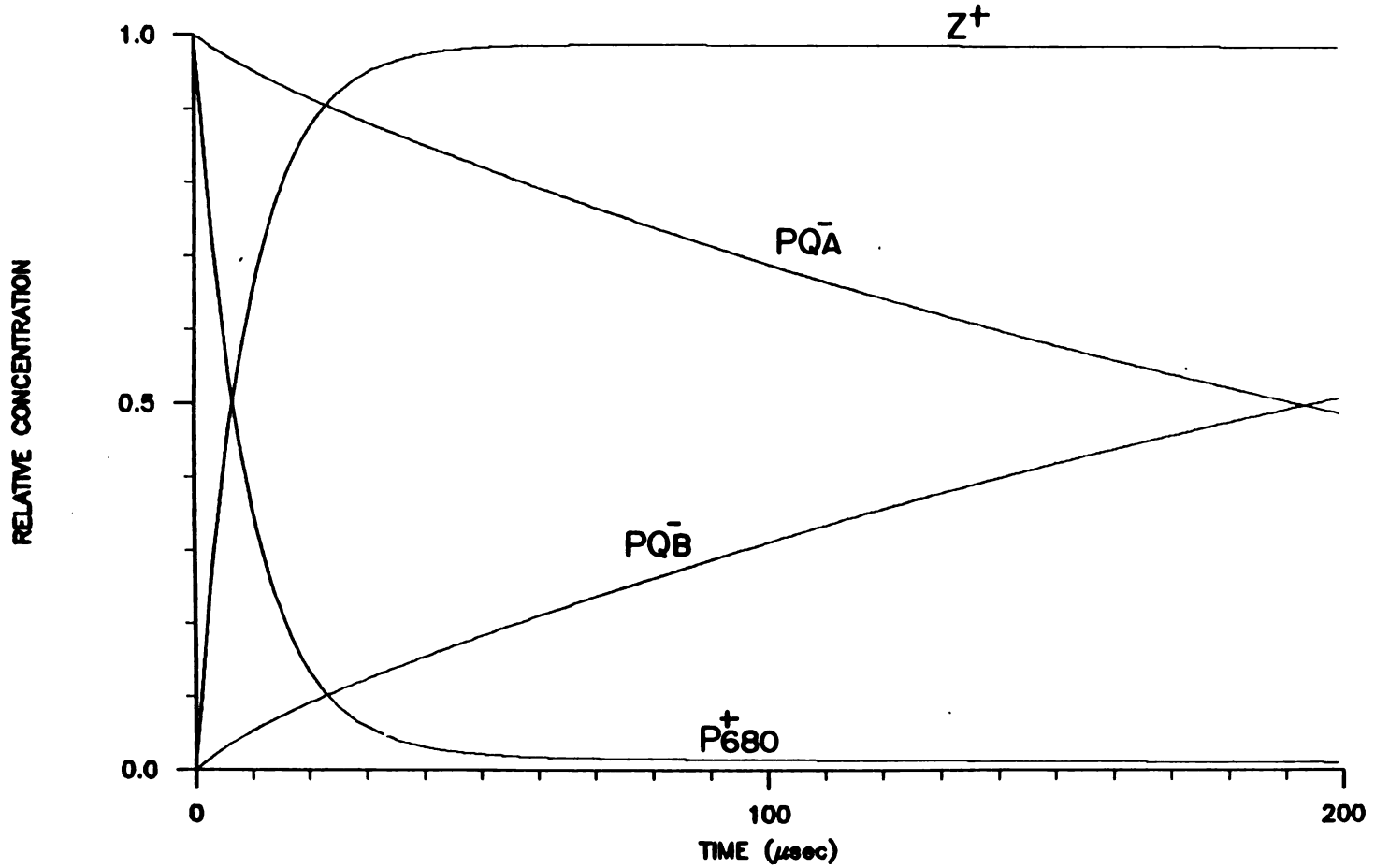
Figure V-1 shows simulations of $P680^+$ (X1), PQ_A^- (X1 + X2), Z^+ (X2 + X4), and PQ_B^- (X4) as a function of time for tris-washed chloroplasts at pH 7.0 as predicted from Eq. (V-4). In Fig. V-1, k_1, k_b, and k_2 were obtained from published values in the literature and correspond to halflives of 7 [31], 120 [69], and 200 [26] μs, respectively. Attempts to determine k_{-1} by regression analysis using d.l. decay curves were not successful, but based upon an approximate 100 mV difference in midpoint potential between Z and $P680^+$, k_{-1} was estimated to correspond to a halflife of 320 μs. Although this is an extremely rough estimation for k_{-1}, the agreement between the concentration profile of Z^+ shown in Fig. V-1 and

FIGURE V-1

Plot depicting the time dependence of the various PSII components in tris washed chloroplasts as predicted by Equation V-4. k_1, k_b, and k_2 were obtained from published values and corresponded to halflives of 7 [31], 120 [69], and 200 [26] μs, respectively. k_{-1} was estimated as described in the text and corresponded to a value of 320 μs.

PSII ELECTRON TRANSPORT PROFILE

Tris–EDTA washed chloroplasts
dark adapted
simulation



Signal IIf in the 0 to 200 μs time range (see Fig. V-2) indicates that the $ZP680^{+} \longleftrightarrow Z^{+}P680$ equilibrium lies to the right and that oxidation of P680 by Z^{+} does not significantly affect μs PSII electron transport behavior. The concentration profile for PSII constituents in tris, DCMU inhibited chloroplasts is shown in Fig. V-3. The major difference between Fig. V-3 relative to V-1 is that the lifetime of PQ_A^{-}, which is only oxidized by a back reaction with $P680^{+}$, is considerably longer.

The precursor to d.l. is $P^{+}Q^{-}$ and therefore the instantaneous intensity of d.l. should be proportional to Xl. A comparison of Figures III-3 and III-4 to Figures V-1 and V-3 demonstrates that the simple electron transport model to describe d.l. is inadequate. Although there is a good correlation between d.l. and Xl ($P^{+}Q^{-}$) at times in which the fast phase of d.l. is dominant, deviation between experimental results and theoretical predictions become apparent even at intermediate times following initiation of photochemistry. A major cause for this discrepancy might arise from uncertainties in the experimental determinations of the rate constants used in the simulation. Although the halflife of the reduction of $P680^{+}$ by an endogenous donor (Z) has been measured optically [31], by using ESR [40], and from fluorescence induction experiments [96] to be about 7 μs for tris-washed chloroplasts (pH 7.0), there exist extensive reports in the literature of the existence of a 35 to 50 μs phase for the reduction of

FIGURE V-2

Kinetics for the formation of Z^+ as measured by the rise of the ESR Signal IIf [40].

SIGNAL IIf RISE KINETICS
Enriched PS II Fragments
Tris-EDTA washed
pH 6.9

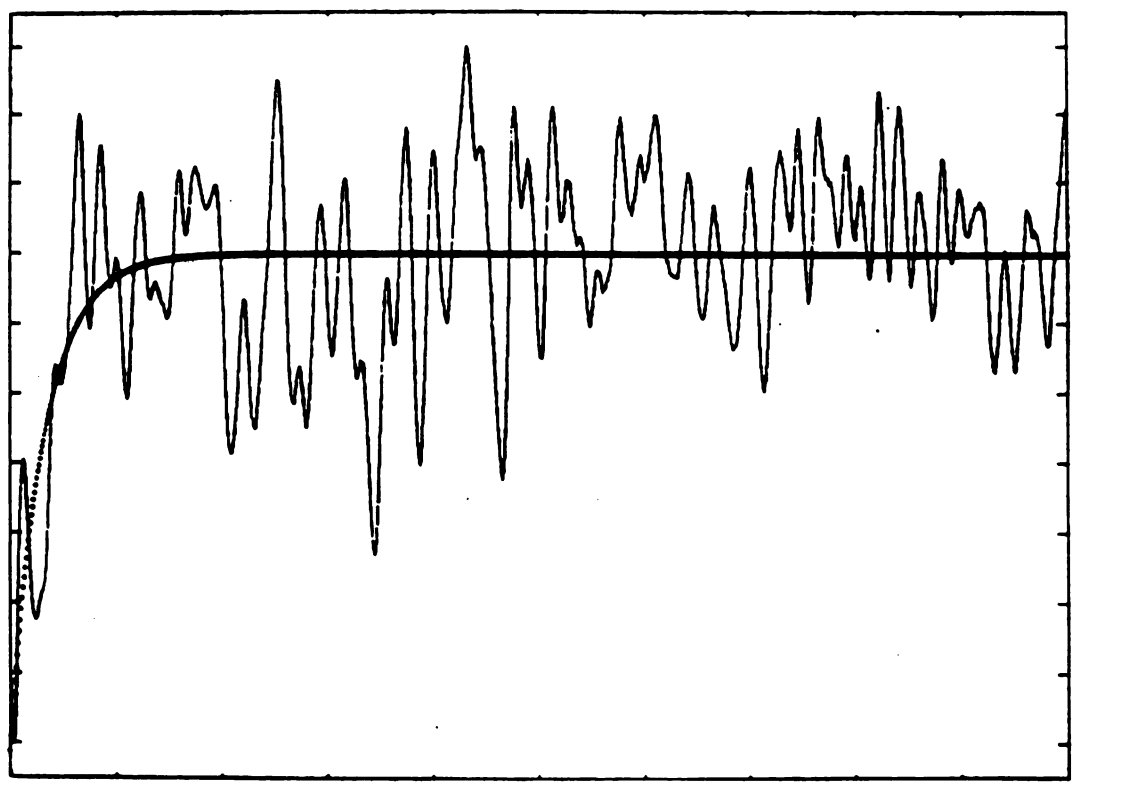
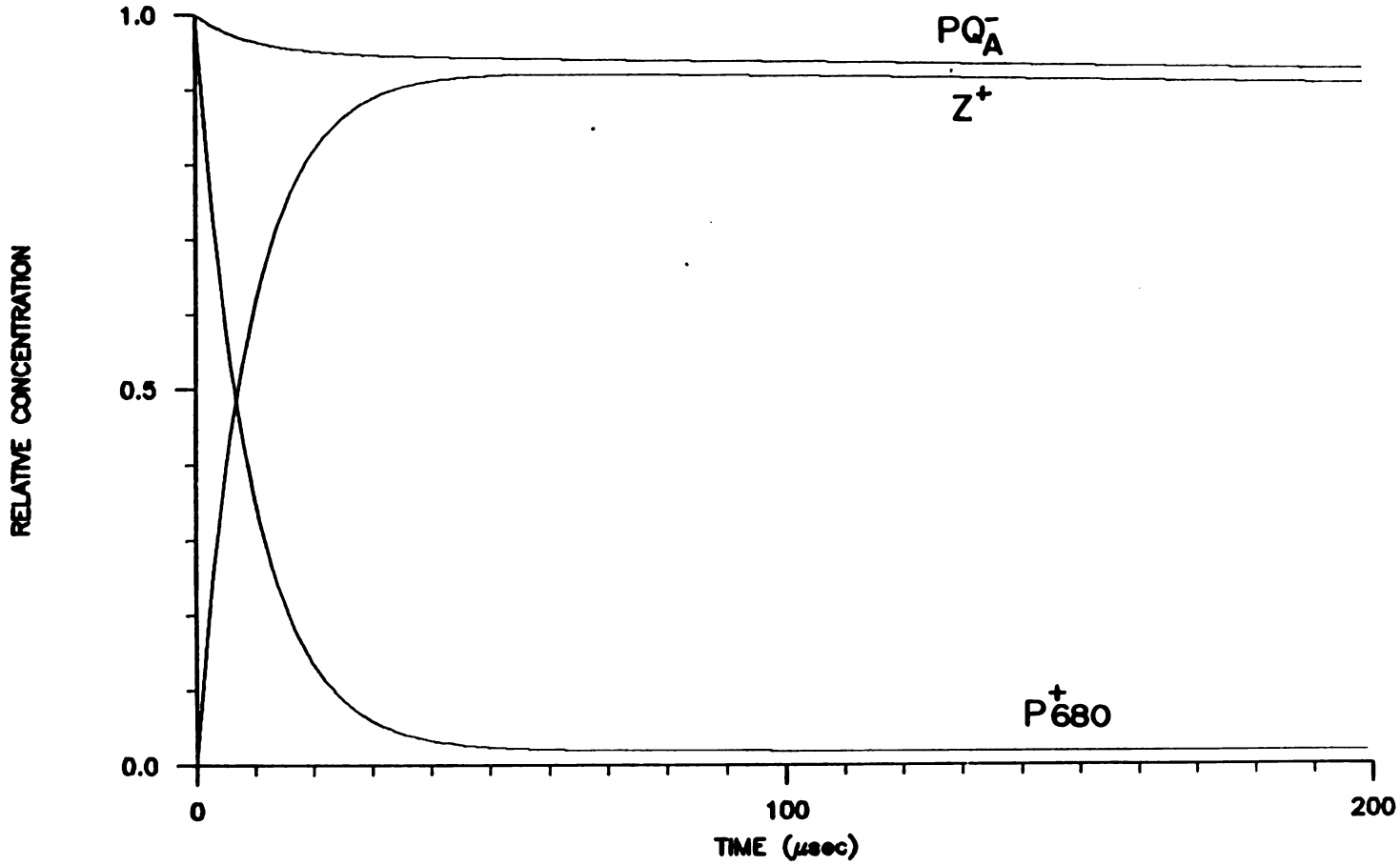


FIGURE V-3

Plot depicting the time dependence of the various PSII components in tris washed, DCMU treated chloroplasts as predicted by Equation V-4. All conditions are identical to those of Figure V-1 except for $k_2 = 0$.

PSII ELECTRON TRANSPORT PROFILE

DCMU, Tris–EDTA washed chloroplasts
dark adapted
simulation



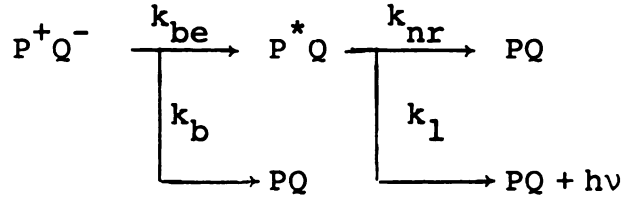
$P680^+$. The experimental techniques used to detect this phase include optical [31], fluoresence [97], and delayed luminescence studies [82]. No evidence for the existence of a 35 μs phase was observed in the Signal IIf rise experiments (see Fig. V-2). However, these experiments were the average of 40000 to 50000 measurements performed under repetitive flash conditions in the presence of exogenous electron donors and acceptors in order to facilitate dark relaxation of the system. The resulting signal/noise ratio was still quite low and it is possible that multiphasic behavior is obscured. It appears, therefore, that the decay of $P680^+$ is heterogeneous and that it consists of a major decay in the 0 to 10 μs range which is pH dependent and subsequent slower phases in the 20 to 100 μs range which are also pH dependent. It is quite possible that the biphasic behavior of delayed luminescence in the 7 to 200 μs time range arises from a structural heterogeneity in PSII [98]. In optical studies involving highly enriched PSII particles, $P680^+$ was reduced by a dominant 10 μs phase. However, a significant fraction of $P680^+$ was observed to decay with slower multiphasic kinetics. Subsequent investigations of the generation of Z^+ as monitored by ESR have shown that in these particles, only a fraction of $P680^+$ is reduced by Z. This fraction was approximately equal to the relative contribution of the 10 μs phase for the total $P680^+$ decay [52]. Apparently, in a fraction of PSII centers, electron transport

between P680 and Z is inactivated; the 35 to 50 μs phase of d.1. might reflect $P680^+$ reduction in these centers.

Although the 35 to 50 μs phase of d.1. cannot be conclusively assigned to specific PSII phenomena, the 10 μs phase is an accurate indicator of the reduction of $P680^+$ by Z. Therefore, emphasis will be placed on this aspect of d.1. In untreated chloroplasts, $P680^+$ reduction by Z proceeds with submicrosecond kinetics, thus the 10 μs phase of d.1. which is observed in untreated chloroplasts, is probably due to a fraction of PSII centers which are damaged and have electron transport to the OEC inhibited. It is apparent from the identical light saturation behavior of the two submillisecond phases of d.1. (see Fig. III-5) that PSII heterogeneity as measured by d.1. cannot be attributed to heterogeneity of the quinone acceptor (Q_α and Q_β) described in [86] because the photoinduced reactions associated with Q_α and Q_β have distinctly different light saturation behavior.

A more serious inadequacy of the simple electron transfer model of delayed luminescence, however, is its inability to predict relative yields of d.1. Under conditions in which electron transport has been significantly perturbed, such as through tris-washing, the back reactions should begin to dominate, especially after several turnovers. However, a comparison of the observed intensity of d.1. for untreated chloroplasts relative to that observed in tris-washed samples (see Tables III-2 and 3

and reference 83) indicates that only slight changes in d.l. yield occur, and in fact, under certain conditions that d.l. intensity from untreated chloroplasts is greater than that from tris-washed samples. Although the 10 μs phase of d.l. in untreated chloroplasts may arise from damaged centers, based upon oxygen evolution rates of 200 to 250 μmole O_2/mg_{chl} hr, these damaged centers would represent only a small percentage (perhaps 5 to 10%) of the total PSII centers. In order to consider explicitly factors which affect the quantum yield of d.l., it is necessary to investigate the generation, by back reaction, and subsequent fate of an exciton in PSII.



(V-6)

where k_{be} is the rate constant for the back reaction resulting in the generation of an exciton and k_b is the rate constant for the parallel back reaction which generates the ground state reaction center. k_{nr} and k_l are the rate constants for the total nonradiative decay and radiative decay of the exciton, respectively. Because the relaxation of the exciton occurs much faster than its generation, it was not necessary to explicitly consider k_l and k_{nr} in the scheme represented by Eq. (V-1). The overall yield of d.l. would then simply be the product of

the probability of generating an exciton ($k_{be} \times [P^+Q^-]$ and the probability that the exciton relaxes in a radiative manner [81]:

$$d.l. = k_{be}\left(\frac{k_l}{k_l+k_{nr}}\right)[P^+Q^-] \qquad (V\text{-}7)$$

It has been proposed that since the extrapolated d.l. intensity is strongly temperature dependent but that the net rate of the back reaction is essentially temperature independent, $k_b >> k_{be}$ [99]. Hence, the rate of P^+Q^- back reaction is determined primarily by k_b. Thus changes in d.l. intensity would merely reflect changes in k_{be}, i.e., changes in the exciton yield; changes in k_{be}, however, would not significantly affect the net rate of the back reaction. If k_{be}, k_{nr} and k_l were constant, the d.l. kinetic behavior and yield would be directly related to the electron transfer reaction of PSII. It is apparent, however, that the d.l. intensity in the 7 to 200 μs time range from tris-washed chloroplasts, in which the $P680^+$ reduction time is about 7 μs, relative to untreated samples, in which $P680^+$ is reduced in less than 1 μs, is much less than would be expected. This difference could possibly arise from either changes in the P^+Q^- exciton recombination yield or in the fluorescence quantum yield once the exciton is formed.

As mentioned in the introduction, $P680^+$ is a quencher of fluorescence [61]. It is generally assumed that $P680^+$ is

as efficient a quencher of fluorescence as PQ_A^- [100]; the fluorescence intensity with PQ_A reduced is about 3 to 5 times that observed in dark adapted chloroplasts. Unlike prompt fluorescence, the chemical generation of an exciton by P^+Q^- recombination results in the depletion of the $P680^+$ concentration. Thus, if the exciton remains localized around the reaction center of its origin, the quenching effect of $P680^+$ on d.l. would not be significant. However, fluorescence studies have indicated that excitation energy absorbed by the antenna system associated with a closed reaction center (PQ_A reduced), may induce photochemistry in a neighboring center [101]. Thus excitons tend to delocalize throughout the antenna system. Butler [103] derived an expression for the fluorescence yield for the connected packaged model of PSII, and through a comparison with experimental results, obtained estimates for the various parameters. Modifying Butler's expression to incorporate the chemical generation of an exciton as opposed to the photogeneration of an exciton, and the quenching effect of $P680^+$, the d.l. behavior would be:

$$d.l. = \frac{\psi_{F11}(1-\psi_{T11}\psi_{t11}{}^{A})}{1-\psi_{T(22)}-\psi_{T11}\psi_{t11}+\psi_{T(22)}\psi_{T11}\psi_{t11}{}^{A}} k_{be}[P^+Q^-] \qquad (V-8)$$

In Eq. (V-8), $\psi_{T(22)}$ is the connecting parameter between PSII units, ψ_{F11} is the intrinsic fluorescence yield, and $\psi_{T11}\psi_{t11}$ represents the product of the probability of

exciton trapping by the reaction center and the probability of the return of the exciton from a closed reaction center to the antenna system. Equation (V-8) assumes a similar behavior for the fluorescence yield and the luminescence yield. Originally A represented the fraction of open centers. In the simulations described below and shown in Fig. V-4, the exciton quenching effect of $P680^+$ is incorporated by allowing A to consist of the sum of the fraction of centers with PQ_A oxidized and the centers with P680 oxidized. This effect is most pronounced in tris-washed chloroplasts in which all of the reaction centers are reduced with 10 μs kinetics. In untreated chloroplasts, $P680^+$ is reduced mainly by submicrosecond kinetics, and the 10 μs phase occurs primarily in damaged centers. Figure V-4 depicts the relative luminescence yields and d.l. intensity for tris-washed chloroplasts and untreated chloroplasts assumed to have 5% or 20% damaged centers. The quenching effect of $P680^+$ on d.l. is quite significant. With only 20% of the PSII centers inhibited, the initial d.l. intensity is over 60% of that observed for tris-washed chloroplasts. Thus $P680^+$ may serve both as a precursor and quencher to d.l. It is apparent from Fig. V-4, that the explicit consideration of the quenching effect of $P680^+$ explains, in part, the origin of the nearly identical d.l. intensity in untreated and tris-inhibited samples. However, other factors exist which affect relative d.l. intensity. For example, the 10 μs phase of d.l. in

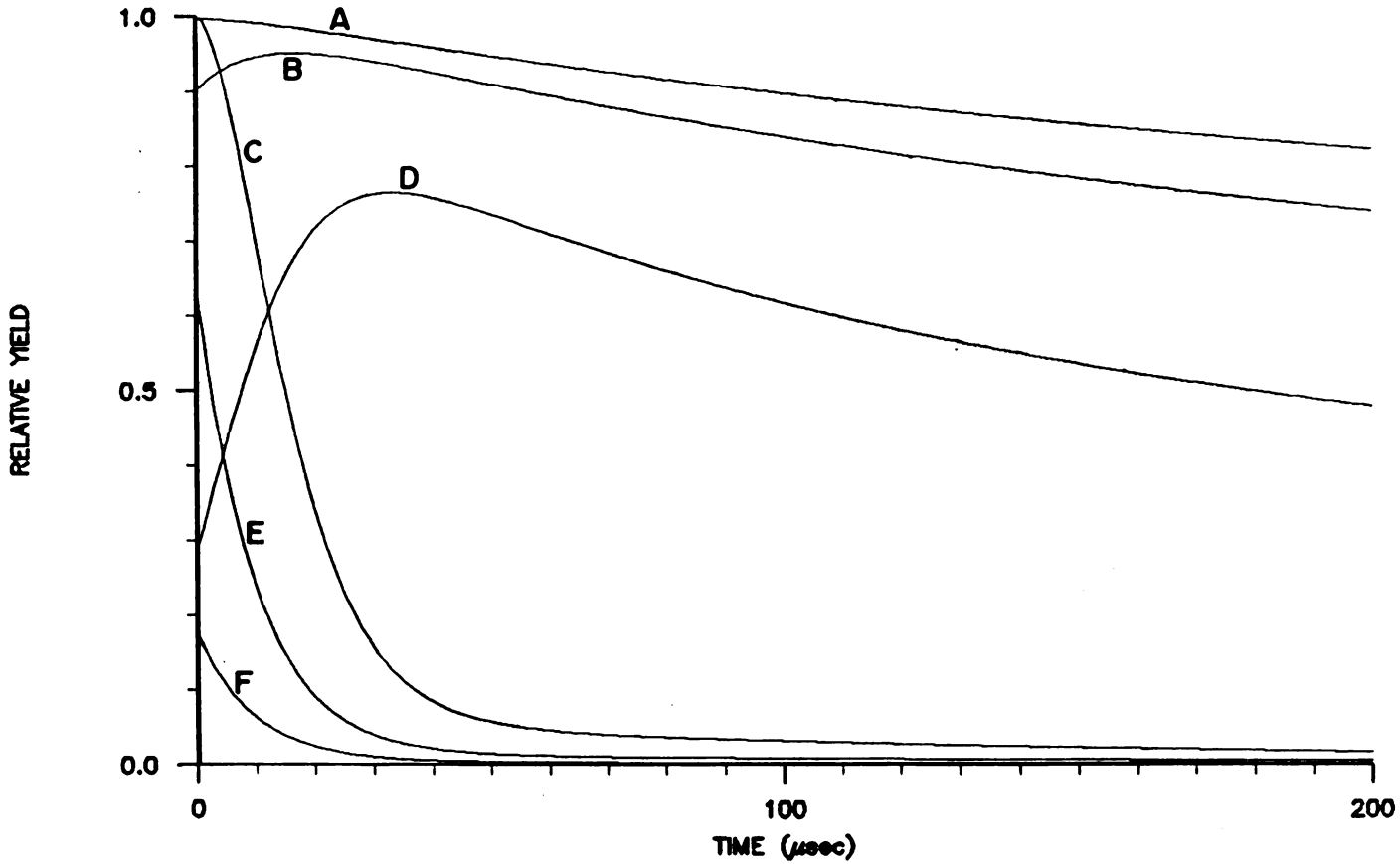
FIGURE V-4

Delayed luminescence and luminescence yield behavior as predicted by Equation V-8 for tris-washed chloroplast and untreated chloroplasts with a fraction (0.05 and 0.20) of damaged centers. The values for the various parameters were $\psi_{t(22)} = .57$, $\psi_{T11}\psi_{t11} = .31$. $[P680^{+}PQ_{A}^{-}]$ was calculated as described in Figure V-1.

A) Relative luminescence yield for untreated chloroplasts (5% damaged).
B) Relative luminescence yield for untreated chloroplasts (20% damaged).
C) Relative d.l. intensity for tris-washed chloroplasts.
D) Relative luminescence yield for tris-washed chloroplasts.
E) Relative d.l. yield for untreated chloroplasts (20% damaged).
F) Relative d.l. yield for untreated chloroplasts (5% damaged).

FLUORESCENCE YIELD AND D.L. PROFILE

Tris–EDTA and Untreated (0.05 and 0.20 damaged) simulation



untreated chloroplasts is most likely due to damaged centers, yet d.l. intensity displays a distinct flash oscillatory behavior (see Figs. III-14 and 15). Although this observation is usually invoked to argue for the physiological significance of the fast phase of d.l. in untreated samples, it is shown in Fig. III-16 that a significant fraction of the PSII centers may be damaged (up to 35% as measured by steady state oxygen yield) before flash induced oscillations in d.l. are eliminated. It was suggested in Chapter 3 that the flash oscillations in d.l. intensity are a reflection of changes in the local membrane potential associated with the sequential release of protons.

CHAPTER 6

DISCUSSION

It is obvious that delayed luminescence is a PSII phenomenon. D.l. is, however, an indirect probe of PSII and it is possible that d.l. is actually a monitor of PSII events which occur in only a minority of centers. Yet, certain aspects of d.l. are clear. The identical pH dependent kinetics for the fast phase of d.l. and the reduction of $P680^+$ in tris-washed chloroplasts and enriched PSII particles [84] verifies that the fast phase of d.l. arises from $P680^+$ reduction. Moreover, based upon the complimentary measurements on $P680^+$ decay and Signal IIf rise as monitored by ESR [40], this phase of d.l. reflects $P680^+$ reduction by Z.

The experiments of Chapter 3 were not able to elucidate a specific cause for the origin of the pH dependence. It was postulated that the pH dependence was related to an acid-base equilibrium of Z. However, as pointed out, a simple protonation/deprotonation reaction of the $P680^+$ donor does not account for the total behavior, particularly that observed at the extreme pH ($pH > 4.5$ or $pH < 8.5$). Furthermore, it was conclusively demonstrated that the kinetics associated with the reduction of $P680^+$ are not

significantly affected by the bulk membrane potential (see Figs. III-8, 9, and 10). Thus it appears that the pH dependence of the rate of $P680^+$ reduction arises from local pH dependent electrostatic interactions. The origin of the electrostatic interaction could result from proximal acid-base groups (such as OH/O^- or NH_3^+/NH_2). A similar model was invoked to explain pH dependent electron transport on the reducing side of P870. Specifically, further reduction of the semiquinone form of the secondary quinone (UQB) was impeded at high pH [103].

The electron transfer model for d.l. described in Chapter 5 adequately accounted for the fast phase of d.l. in tris-washed chloroplasts. This model, however, was not able to account for the 35 (35 to 65) μs phase. Even when the model was refined to accommodate factors which affect the overall d.l. yield, the origin of the 35 μs phase was still obscure. Because of the extensive reports in the literature which verify the existence of a 35 μs phase of $P680^+$ reduction [36,82,97] it was argued that this phase represented a heterogeneity in P680. This is substaniated by Eckert and Renger [104] who demonstrated that the oxidation equivalents generated by the fraction of $P680^+$ which is reduced with a 35 μs halflife are not available for water oxidation. The fact that a heterogeneity exists should not be surprising. It is likely that within the leaf, there exists a distribution of PSII centers at various stages of development. The procedure to

isolate chloroplasts from whole leaves is nondiscriminatory. Thus PSII centers which do not have fully functional electron transport chains would be obtained along with those that are fully functional. Therefore, the heterogeneity may arise from either immature or aged centers, as well as from centers which are damaged during isolation. The identical light saturation behavior in dark adapted tris-washed chloroplasts of the 7 and 50 μs phase (see Fig. III-5) indicates that the antenna system is fully developed for both populations of the centers. This does not argue against heterogeneity since physiological studies have demonstrated that the ability to oxidize water represents part of the final statge of chloroplasts development [105].

The existence of the 7 to 10 μs phase in oxygen evolving chloroplasts which oscillates with a period four is perplexing. The currently accepted model for PSII electron transport in intact centers does not accommodate a 10 μs phase. However, there do exist several reports in the literature describing the existence of a 10 μs phase for $P680^+$ reduction. Mathis and coworkers [36] reported that the amplitude of the 10 μs phase of absorption change associated with oxidized P680 in untreated chloroplasts was about 60% of that observed for tris-washed samples. In a subsequent investigation in which a detection apparatus with improved time resolution was used, however, it was shown that $P680^+$ decayed primarily by submicrosecond kinetics and the slower phases present represented only a

small but unspecified fraction of the total $P680^+$ pool [34]. It thus appears that the 10 μs phase of d.l. is due to a heterogeneity of P680 and might arise from centers with inactive electron transport from the OEC. An ambiguity occurs however, in these optical studies in that the signal attributed to $P680^+$ at 820 nm potentially has contributions from $P700^+$. Although interferences from $P700^+$ can be minimized by the addition of ferricyanide and illumination by far red light to maintain P700 in the oxidized form, it is possible that some of the absorption changes attributed to $P680^+$ may in actuality arise from PSI. Optical studies using PSII fragments capable of generating oxygen [71] should help remove this ambiguity. Delayed luminescence however, has negligible interference from PSI.

The intensity of d.l. (in the 7 to 200 μs time range) from tris-washed and untreated samples differs only slightly. To account for the similarity in d.l. yield in intact chloroplasts and that of tris-washed chloroplasts, it becomes apparent that the overall luminescence yield is greater in intact samples than in tris-washed chloroplasts. This difference in d.l. yield between intact and tris-inhibited chloroplasts may be quite significant since the 10 μs phase of d.l. in oxygen evolving samples probably originates from inactive centers and from the fact that the majority of $P680^+$ (estimated to be >90%) is reduced in less that 1 μs. Part of the apparent discrepancy of d.l.

intensity can be explained by the well documented exciton quenching effect by $P680^+$. Incorporation of this effect by utilizing the model described by Eq. (V-8) results in a significant decrease in d.l. intensity in tris-washed chloroplasts. Although this model does not completely explain the low d.l. intensity in tris-washed chloroplasts relative to that observed for intact samples, it represents the first explicit consideration of the role of $P680^+$ as a quencher of d.l. Additionally, it addresses directly the cause for the low yield of d.l. under conditions in which it would intuitively be thought to be high. Finally, it provides an explanation for the anomalous 10 μs phase of d.l. in "intact" chloroplasts and thus rationalizes d.l. in terms of well characterized electron transfer components. Failure to do so in the past has resulted in the introduction of additional electron transfer components in PSII. The results presented above allow us to eliminate these additional components while retaining a cogent and coherent explanation for μs d.l. Other factors which might affect d.l. yield, such as flash induced changes in membrane potential were described in Chapter 5. These changes in local membrane potential could be related to the release of protons in oxygen evolving centers. The proton release pattern reported by Fowler [90] would predict a maximum charge stored on the oxidizing side of P680 just prior to the third flash. The results in Figs. III-8 through III-10 indicate that factors which affect the membrane potential

also affect the intensity but not necessarily the kinetics of d.l. The addition of divalent cations (Ca^{2+}) resulted in an quenching of d.l. and as previously indicated, with 1.0 M Ca^{2+} the quenching was nearly complete. Monovalent cations (K^{+}), however, had only a slight effect on d.l. behavior. Moreover, the d.l. intensity from samples treated with gramcidin was 50 to 75% of the intensity observed in the control without an appreciable effect on the kinetics indicating the role of the membrane potential on the overall d.l. yield. It is possible, however, that the gramicidin effect is on the bulk membrane potential and that local fields are not affected which would account for the four flash oscillatory behavior of d.l. in the presence of gramicidin (see Fig. III-10).

Changes in d.l. intensity, but without accompanying changes in kinetics, are often cited as evidence for changes in the rate constants for the various electron transfer reactions of PSII. An important point about the results of Chapter 5 is that the intensity of d.l. is a poor indicator of the rate of electron transfer, and that the simple electron transfer model for d.l. cannot accommodate changes in d.l. intensity without changes in kinetics. An inspection of Eq. (V-4) will indicate that both the pre-expotential factors and the time constants are functions of the various rate constants. Therefore, within the constraints of the simple electron transfer model for d.l., it is impossible to alter the d.l. yield without affecting

the kinetics. As indicated by Eq. (V-7), changes in the d.l. intensity reflection of changes in the overall luminescence yield and is not related to electron transfer rates.

The EPL technique offers promise as a probe for the oxidizing side of PSII; the invariance of the kinetic behavior with flash number of EPL in tris-washed chloroplasts indicates an insensitivity of EPL to reducing side events. Additionally, a kinetic analysis of EPL in the presence of PSII donors (see Figs. IV-8 and IV-9) gives essentially the same results as those obtained from ESR studies on Z^+ reduction. This demonstrates the role of Z as a precursor to EPL. This experiment indicates that the EPL effect arises from an electric field induced reversal of electron transfer reaction. This is further substaniated by results shown in Fig. IV-12 in which the ratio EPL/d.l. is plotted. The time dependence of this enhancement seems to indicate a greater effect on destabilizing the luminescence precursor at longer dark times than at short times. Additionally, the flash number dependent kinetics of EPL in untreated chloroplasts follows the same behavior as that observed for Signal IIvf. This indicates that the induced luminescence from oxygen evolving samples originates from intact centers, whereas the unperturbed d.l. appears to originate primarily from damaged centers.

The sensitivity of EPL to events on the oxidizing side of PSII indicates that the electric field induced enhancement of luminescence arises primarily from the destabilization of intermediate states of PSII. The effect of the induced electric field is hard to quantify. The cos(θ) dependence (see Fig. IV-4) demonstrates that not all centers within the thylakoid membrane are perturbed to the same extent. Furthermore, there exists a fairly wide distrubtion in bleb sizes [93] which results in differences in the magnitude of the maximal induced field. Thus, although the applied field is homogeneous, the induced field has a distribution of values and it is this transmembrane field which destabilizes the intermediate states of PSII. The situation is further complicated by a heterogeneity in PSII. This would not only include the intrinsic heterogeneity in centers, such as those that give rise to the 35 μs phase of $P680^+$ reduction, but also the difficulty in applying the field while PSII is in a specific state. In dark-adapted, tris-washed chloroplasts at pH 5.2, the halflife for the reduction of $P680^+$ is about 17.9 μs. Thus for a field applied 15 μs after illumination, about 60% of the centers are in the $P680^+PQ_A^-$ state and 40% in the $Z^+PQ_A^-$ state. The results of Figs. IV-13 through IV-20 probably reflect the difficulty in controlling the effective perturbation.

Although the model depicted in Fig. IV-7 is qualitatively correct, a quantative analysis is not feasible.

Extremely useful results, however, are still obtainable from the EPL technique. The induced back reaction between $P680^+$ and PQ_A^- substantiates the vectoral nature of primary electron transport [43]. Furthermore, the extreme sensitivity of EPL to Z^+, as indicated by the effect of donors (Figs. IV-8 and IV-9), the flash number dependent kinetics in untreated chloroplasts (Fig. IV-10), and the flash number independent kinetics in tris-inhibited chloroplasts (Fig. IV-11) demonstrate the field induced oxidation of P680 by Z^+. This argues that relative to P680, Z is situated towards the inside of the membrane. Additionally, the insensitivity of EPL to reducing side events indicate that within the membrane, PQ_A and PQ_B are essentially tangential to each other. These results are incorporated in Fig. I-3 depicting the relative orientation of various PSII components. In Fig. I-3, the site of water oxidation is depicted as being sequestered within the membrane; specifically it is isolated from the internal aqueous phases by various protein subunits. Evidence for this orientation was obtained from the behavior of EPL in untreated chloroplasts. The flash number dependence in untreated chloroplasts for the EPL effect is not observed in the millisecond time range. This insensitivity of EPL on the S states indicates that the site of the stored equivalents necessary for water oxidation are not on the inside surface of the membrane, i.e., P680 and Z are approximately tangential to the OEC.

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