PHOTOSYSTEM II CHARGE STABILIZATION REACTIONS IN ISOLATED CHLOROPLASTS

BY

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THESIS

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WE HEREBY RECOMMEND THAT THE THESIS BY

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D517

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ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
D	Secondary electron donor to the Photosystem II reaction center
DCMU	3-(3',4'-dichlorophenyl)-1,1'-dimethyl urea
E_a	Activation energy
EDTA	Ethylenediamine tetraacetic acid
ESR	Electron spin resonance
FeCN	Ferricyanide
$F_m(t)$	Chlorophyll a fluorescence intensity at time t
F_0	Chlorophyll a fluorescence intensity prior to excitation
ΔG	Change in free energy
GMCD	Gramicidin D
$h\nu_{DLE}$	Quantum of delayed light emission
$h\nu_{II}$	Photon absorbed by Photosystem II
I	Light intensity
I_{DLE}	Intensity of delayed light emission
L'	Delayed light emission intensity with a salt-jump
L	Delayed light emission intensity without a salt-jump
L_c	Portion of delayed light emission intensity that is insensitive to membrane potential
$L\psi$	Portion of delayed light emission intensity that is sensitive to membrane potential
M	Oxygen evolving complex
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

NH_2OH	Hydroxylamine
NIG	Nigericin
12NS	12 nitroxide stearate
P_i	Inorganic phosphate
P_{680}	Photosystem II reaction center
P_{700}	Photosystem I reaction center
Q	"Primary" electron acceptor of Photosystem II
Q_1, Q_2	Two different states of the primary electron acceptor of Photosystem II
SiMo	Silicomolybdate
S_n	Charge accumulator for the oxygen evolving system, where n = 0, 1, 2, 3, 4 reaction steps
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
TRICINE	N-tris(hydroxylmethyl)methylglycine
TRIS	Tris(hydroxymethyl)aminomethane
$2\ T_{11}$	Hyperfine splitting constant of electron spin resonance spectra
VAL	Valinomycin
W	Proposed electron acceptor between P_{680} and Q
Z, Z_1	First primary electron donor to the Photosystem II reaction center
Z_2	Second primary electron donor to the Photosystem II reaction center
α	Exponential component amplitude
τ	Exponential lifetime
$\tau_{\frac{1}{2}}$	Halftime
τ_c	Rotational correlation time of electron spin resonance spin labels
ϕ_{DLE}	Delayed light emission yield

$\phi_{DLE}^{6\ \mu s}$	Yield of 6 μs delayed light emission component
$\phi_{DLE}^{50\ \mu s}$	Yield of 50 μs delayed light emission component
ϕ_f	Chlorophyll a fluorescence yield
$\phi_f(max)$	Maximum chlorophyll a fluorescence yield
ϕ_0	Chlorophyll a fluorescence yield prior to excitation
ψ_D	Portion of membrane potential that effects delayed light emission
ψ_l	Light generated membrane potential
ψ_m	Total membrane potential across the thylakoid
ψ_s	Salt-jump generated membrane potential

CHAPTER I

GENERAL INTRODUCTION

A. Photosynthesis

Green plant photosynthesis uses absorbed light energy to drive a number of oxidation reduction reactions which lead to the oxidation of water to molecular oxygen and the reduction of carbon dioxide to carbohydrates [1]. The photosynthetic pigments and the redox carriers are associated with closed membraneous disks called thylakoids located in the chloroplast (higher plants and most algae) or in the cell (blue-green algae) [2]. Approximately 200-300 chlorophyll a molecules act together as a light absorbing unit and cooperatively transfer absorbed light energy to a special reaction center site where the initial chemical oxidation reduction reaction takes place. These pigment groups are called photosynthetic units of which two types are known: (a) Photosystem I containing relatively more long wavelength absorbing forms of chlorophyll a and a reaction center chlorophyll a designated as P_{700}, and (b) Photosystem II containing relatively more of short wavelength absorbing forms of chlorophyll a and a reaction center chlorophyll a designated as P_{680}. The two photosystems operate in series with Photosystem II oxidizing water and producing a weak reductant and Photosystem I oxidizing this weak reductant and producing the strong reductant, reduced nicatinamide adenine dinucleotide phosphate (NADPH). Associated with the transfer of electrons from Photosystem II to Photosystem I is an energetic coupling to a phosphorylating mechanism which generates adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i). The NADPH and ATP drive the reactions which fix carbon dioxide and produce carbohydrates.

B. Photosystem II

The proposed reactions of Photosystem II are as follows:

$$ZP_{680}Q \xrightarrow{h\nu_{II}} ZP^{+}_{680}Q^{-} \qquad (1)$$
(ϕ_f low) (ϕ_f low)

$$ZP^{+}_{680}Q^{-} \longrightarrow Z^{+}P_{680}Q^{-} \qquad (2)$$
(ϕ_f low) (ϕ_f high)

$$Z^{+}P_{680}Q^{-} \xrightarrow{S_n\ S_{n+1}} ZP_{680}Q^{-} \qquad (3)$$
(ϕ_f high) (ϕ_f high)

Intersystem Electron Carrier Pool

$$ZP_{680}Q^{-} \xrightarrow{e^{-}} ZP_{680}Q \qquad (4)$$
(ϕ_f high) (ϕ_f low)

where Z is the electron donor to the reaction center chlorophyll *a* P_{680}, Q is the "primary" electron acceptor, $h\nu_{II}$ photon absorbed in pigment system II, ϕ_f is the quantum yield of fluorescence, and S_n is the charge accumulator for the oxygen evolving system.

Reaction 1 is the initial charge separation reaction which occurs in less than 20 ns [3]. In this reaction P_{680} is oxidized [4,5] and Q, a plastoquinone, is reduced to plastosemiquinone [6-8]. In reaction 2, Z reduces the oxidized reaction center chlorophyll, P^{+}_{680} [9]. The Z^{+} oxidizing equivalent is eventually used to evolve O_2 from H_2O. Oxygen evolution [10,11] in chloroplasts or algae, given saturating microsecond flashes, has no yield after the first or the second flash following dark adaptation, maximal yield after

the third flash, and thereafter it oscillates with a period of four. Each Photosystem II reaction center has five oxidation states S_o, S_1,....S_4), with S_4 having four oxidizing equivalents necessary to oxidize two water molecules and evolve one oxygen molecule. Reaction 3 is the advance of the Photosystem II oxygen evolving system by one oxidation equivalent. Reaction 4 is the oxidation of Q^- by the electron carrier pool between the two photosystems. Reaction 2, 3 and 4 return the Photosystem II reaction center complex to its original state ($ZP_{680}Q$) with the result that one photon has caused the net transfer of one electron.

According to Duysens and Sweers [12], chlorophyll a fluorescence yield is quenched by the oxidized but not the reduced form of Q. Thus, by measuring the fluorescence yield decay, reaction 4 can be measured. The half-time of this reaction is a few hundred microseconds [13] and is drastically slowed down upon the addition of 3-(3',4'-dichloropheny) 1,1'-dimethyl urea (DCMU) [12,13]. At shorter times (<50 μs) following illumination, the changes in fluorescence yield cannot be explained without the additional assumption that P^+_{680} is also a quencher of chlorophyll a fluorescence [14]. The rise in fluorescence yield in the microsecond time range following a flash has been interpreted [15] to reflect the disappearance of P^+_{680}. Thus, by observing the rise in chlorophyll a fluorescence yield in the microsecond time range reaction 2 can be monitored. Reactions 1 and 3 cannot be monitored by fluorescence yield changes as the yield remains low and high, respectively, during these reactions.

C. Delayed Light Emission

In 1951 Strehler and Arnold [16] discovered that a very weak light emission was given off by the intact cells of the green alga Chlorella

minutes after termination of illumination [17]; it has an emission spectrum identical to that of chlorophyll a fluorescence [18, 19, 20]. This suggests that delayed light emission comes from deexcitation of the singlet excited state of chlorophyll a, which is generated in the few nanoseconds to minutes time range from metastable photochemical products. Delayed light emission at room temperature originates mostly from Photosystem II since (a) algal mutants which lack Photosystem II have no measurable delayed light emission [20, 21], (b) action spectra for Photosystem II activity and delayed light emission are almost identical [19, 20], and (c) Photosystem II enriched particles have a 60 to 90 fold higher delayed light emission than Photosystem I enriched particles [22, 23].

The exact mechanism of delayed light emission generation in photosynthetic systems is unknown. At this time, however, there are three theories which are the following: charge recombination, electron-hole recombination, and triplet fusion.

In the charge recombination theory [17, 20, 24] delayed light emission is generated by the back reaction of the initial charge separation ($ZP^{+}_{680}Q^{-} \longrightarrow ZP^{*}_{680}Q \longrightarrow ZP_{680}Q + h\nu_{DLE}$). Also, delayed light may be generated even if the positive charge is on Z by the reverse charge flow from Z^{+} to produce P^{+}_{680} and then recombination. Support for this theory comes mainly from experiments designed to alter the concentrations of the back reacting components. Delayed light emission, at times greater than 2 ms after termination of illumination, is completely eliminated in samples treated with hydroxylamine (NH_2OH) and DCMU [25, 26]. DCMU stops any loss of Q^{-} except by delayed light emission and NH_2OH causes a reduction of all Z^{+} resulting in reaction centers being in the $ZP_{680}Q^{-}$ state, which are

unable to generate delayed light emission by the recombination process. Delayed light emission at times less than 2 ms is not eliminated in samples treated with NH_2OH since the charge donation time of NH_2OH to Z^+ and P_{680}^+ is longer than 2 ms, allowing Z^+ and P_{680}^+ to back react with Q^- and generate delayed light emission. Silicomolybdate is able to accept electrons directly from Q^- [27, 28]. Its presence was found [28] to greatly inhibit delayed light emission at times greater than 100 ms after termination of illumination, thus, supporting the need for Q^- in the recombination hypothesis of delayed light emission. The charge recombination theory has two problems: (1) the singlet excited state of a chlorophyll molecule requires 1.8 eV of energy, but the calculated energy available from P_{680}^+ and Q^- is at most 1.0 eV [20]; and (2) a linear dependence of delayed light emission on illumination intensity is expected, but a square law dependence is observed [29]. [However, see Chapter VI of this thesis.]

In the electron-hole theory of delayed light emission the photosynthetic unit is thought of as a semiconductor having a conduction band with associated charge traps of various depths. It is believed that there are two sites of trapping in the photosynthetic unit of Photosystem II, one for electrons and the other for holes [30, 31], each having its own reaction center. In the photosynthetic unit, a photon causes an electron and hole to be generated with the electron being trapped at a reaction center and the hole remaining free, while at the other type of reaction center the hole is trapped and the electron would remain free. The recombination of free electrons and holes would generate delayed light emission. The two photons used to generate the above species explains the I^2 dependence of delayed light. This theory does not seem likely at this time since no

evidence exists for the presence of free electrons and holes or the required semiconductor complex.

The triplet theory proposes that upon receiving excitation energy or by Z^+ and Q^- recombination the Photosystem II reaction center may populate triplet states by intersystem crossing [32]. These triplet excitons may decay by radiationless transitions or two triplets may undergo fusion and produce an excited singlet and a ground sate. This excited singlet then decays to the ground state giving rise to delayed light emission. Production of triplet states in green plants has been shown when normal photochemical reactions have been saturated. At high light intensities, when the reaction center is closed to photochemistry, chlorophyll a fluorescence is quenched by the proposed formation of triplets [13, 15]. The presence of triplets was shown by the existence of phosphorescence [33] and by microwave-induced changes in chlorophyll a fluorescence yield at 2° K [34, 35] . Certain thermoluminescence glow peaks have been suggested to arise from metastable triplet states, but only when other reactions are saturated [36]. Thus, although triplet states exist in green plants, there is no evidence, at this time, that triplet-triplet fusion is responsible for delayed light emission in plants exposed to just saturating light.

The charge recombination hypothesis is consistent with the largest portion of experimental results and it will be shown in this thesis that it is the only viable hypothesis for the origin of delayed light emission.

D. Purpose and Scope of the Present Thesis

The main purpose of our experiments was to obtain precise information about the relationship between Photosystem II reactions and delayed light emission in the microsecond time range. To accomplish this, parallel

measurements of chlorphyll a fluorescence yield and delayed light emission were carried out. Single saturating 10 ns excitation flashes were used to excite Photosystem II in order to follow relaxation reactions which are not complicated by decays from earlier excitations. Chapter II describes the apparatus that was developed and the protocol used in making these measurements. Chapters III and IV describe experiments designed to study the effects of alterations in the stabilization reactions which operate on the oxygen evolving side of the Photosystem II reaction center (reaction 2 and 3 above) on both the chlorophyll a fluorescence yield rise and delayed light emission decay. It is shown that in isolated chloroplasts the primary stabilization reaction for the P^{+}_{680} charge (reaction 2 above) has a lifetime of 6 μs and, at least, two charge carriers operate between P_{680} and the oxygen evolving system.

The effects of light and salt-jump generated thylakoid membrane potentials on microsecond and millisecond delayed light are described in Chapter V and from these results, it is suggested that the charge couple (P^{+}_{680} and Q^{-}) responsible for microsecond delayed light has a separation distance transverse to the thylakoid membrane surface of approximately 5 Å and for millisecond delayed light emission approximately 11 Å. In Chapter VI the effects of temperature on microsecond delayed light emission in the 0-35° C temperature range are presented. The 6 μs lifetime decay component of delayed light emission and the 6 μs chlorophyll a fluorescence yield rise are both found to be temperature independent; however, delayed light emission decay kinetics, at times greater than 120 μs after the flash, show variations with temperature which correlate well with the thylakoid membrane lipid fluidity.

Other conclusions will be discussed in individual chapters as will be the special techniques or methods and references pertinent to the specific problem investigated. Major conclusions and a thesis summary can be found in Chapter VII.

CHAPTER II

MATERIALS AND METHODS: GENERAL

A. Preparation of Chloroplasts

Chloroplasts were isolated from leaves of Alaska peas (Pisum sativum, var. Alaska) or bush beans (Phaseolus vulgaris) grown in the laboratory and harvested ten days after seed germination. Also, leaves from spinach (Spinacea oleracea) and lettuce (Lactuca sativa, var. Romaine) obtained from the local market were used. About 75 g of leaves were rinsed in ice water and then homogenized for 20 s in a Waring blender in 150 ml of buffer media consisting of 0.4 M sucrose, 0.1 M N-tris(hydroxylmethyl)methylglycine (TRICINE), 5 mM $MgCl_2$, 10 mM NaCl, and 20 mM ascorbate adjusted to a pH of 7.8. For bean chloroplasts, 250 mg bovine serum albumin was also added to the grinding media. The homogenate was strained through eight layer of cheesecloth and one layer of 10 micron mesh nylon cloth. The filtered liquid was centrifuged at 5000 X g for 5 min to pellet the chloroplasts which were resuspended in a 50 mM sodium phosphate buffer (pH 7.8) to obtain broken chloroplasts. These chloroplast fragments (thylakoids) were pelleted by another 5000 X g, 5 min centrifugation and were finally resuspended to a chlorophyll concentration of approximately 3 mg/ml in a solution identical to the griding media except with 50 mM phosphate buffer instead of TRICINE buffer. Unless otherwise noted all delayed light emission and fluorescence yield measurements were made with samples diluted to a chlorophyll concentration of 5 μg/ml with the phosphate buffer. All chlorophyll concentrations were determined by the method of Arnon [37] using MacKinney's equations [38].

B. Preparation of Algae

Cells of blue-green alga, Anacystis nidulans, were grown at 22 °C in Kratz and Meyer's C medium [39] using previously described procedures [40]. Five day old cultures were used and they were washed and resuspended in a medium containing 0.4 M sucrose, 40 mM KCl and 50 mM phosphate buffer (pH 7.8) prior to use in experiments.

C. Delayed Light Emission Measurements

Delayed light emission was observed in the microsecond time range after a single saturating excitation flash instead of by the phosphoroscope method. The advantages of this method are the following: (1) stabilization steps occurring a few microseconds after the flash can be observed since the excitation lasts only a few nanoseconds instead of tens of microseconds or longer with the phosphoroscope; (2) decays after a single excitation are not complicated by the decay of states generated by previous phosphoroscope cycle excitation; and (3) there is freedom in giving various preillumination regimes.

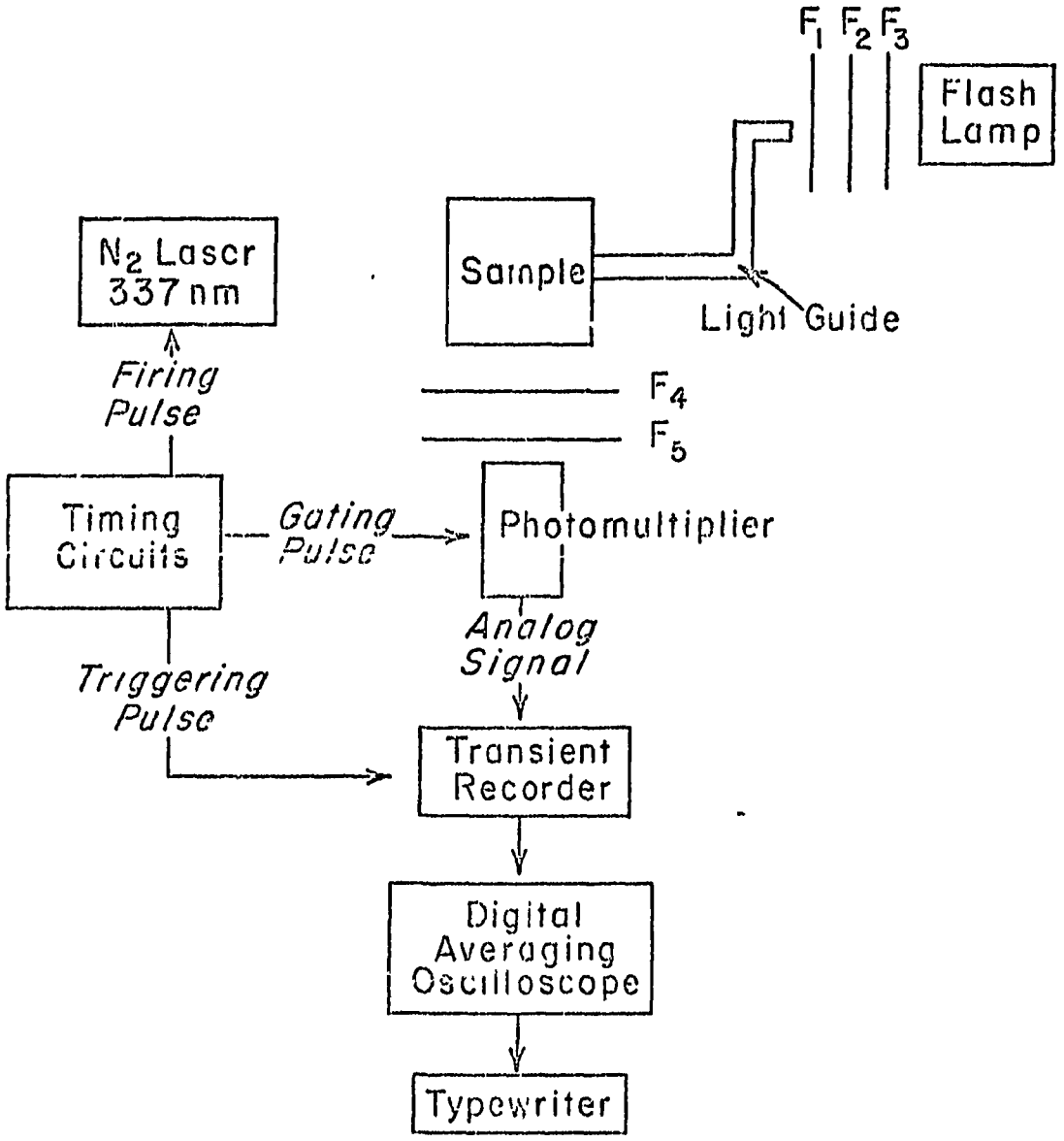
In order to measure delayed light emission in the microsecond time range after an excitation flash the experimental arrangement shown in Fig. 1 was developed. A wide variety of technical difficulties had to be solved in the design and construction of this equipment and the most significant ones will be described below.

1. Excitation Source

The most demanding requirement on the excitation source to be used for the microsecond delayed light emission measurements is that no contribution of the excitation flash exist in the microsecond time range. Since the fluorescence yield of chloroplasts is 10^2 to 10^3 times greater than the yield

Fig. 1. Experimental arrangement for measuring delayed light emission and fluorescence yield rise after an excitation flash.

The sample was held in a 1 cm path length quartz cuvette, the nitrogen laser provided saturating 337 nm, 10 ns actinic flashes, and the flash lamp provided weak analytic flash for the fluorescence yield measurement. The filters used are as follows: F_1 is a neutral density filter, F_2 and F_3 are Corning CS 4-96 (blue), F_4 is a Kodak Wratten 2-A, and F_5 is a Schott RG-8 (red cut off).


F1 F2 F3
Flash Lamp
Sample
Light Guide
N2 Laser 337 nm
Firing Pulse
F4
F5
Timing Circuits
Gating Pulse
Photomultiplier
Analog Signal
Triggering Pulse
Transient Recorder
Digital Averaging Oscilloscope
Typewriter

of delayed light emission, even a low intensity tail from the excitation flash decay will produce a fluorescence signal comparable to delayed light emission. An Avco Everett Model C102 nitrogen laser was, therefore, chosen for excitation (Fig. 1) since nitrogen lasers are self terminating with a 10 ns pulse width at half maximum of the flash. Single 1 mJ pulses of 337 nm emission wavelength were capable of just saturating microsecond delayed light emission and chlorophyll a fluorescence yield in chloroplast samples having chlorophyll concentrations of 5 μg/ml (Fig. 2). Saturation (1 quantum absorbed per photosynthetic unit) occurred at a calculated* intensity level of 1 quantum per 200 chlorphyll molecules and the laser intensity was kept at twice this level in all experiments. Flash intensities higher than this were not used in order to avoid multiple hits of the reaction center which would generate carotenoid triplets. Data analysis becomes more complex when triplets are formed since they quench chlorphyll a fluorescence in the microsecond range. The laser pulse intensity, measured with a JEN-TEC Model ED500 joule meter, was just saturating at an incident intensity of 5×10^{13} quanta/cm^2 equivalent to 1.8×10^{13} absorbed quanta/cm^2, since the percent absorption was 36% at 337 nm.

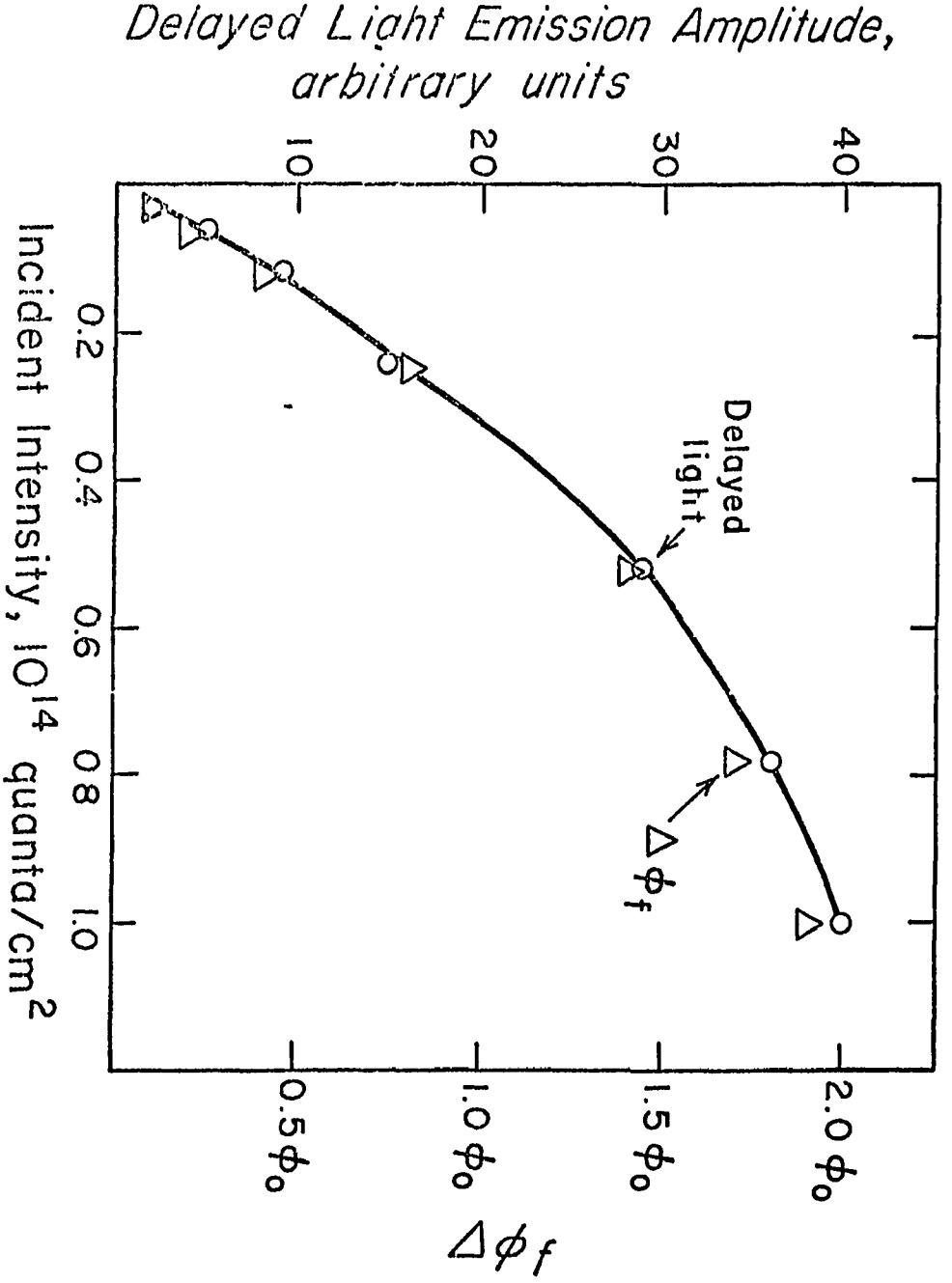
2. Photomultiplier

An Electra Megadyne Inc. 9558B photomultiplier with S-20 cathode was used (Fig. 1) for observing delayed light emission. The rise time of the photomultiplier anode circuit was altered by varying the anode load resistance and the rise time was always adjusted to be at least five times faster than the most rapid decay component being observed in order to

* The intensity at which the rise in the light curve is (1-1/e) = 0.63.

Fig. 2. Light saturation curve for delayed light emission and chlorophyll a fluorescence yield.

Light curve for delayed light emission at 100 μs after a 10 ns excitation flash and the variable chlorophyll a fluorescence yield (ϕ_f) at 20 μs after the excitation flash in units of ϕ_0, the level of chlorophyll a fluorescence yield prior to excitation. Measurements were made on pea chloroplasts at a chlorophyll concentration of 5 μg/ml that had been dark adapted for 5 min. The maximum incident laser intensity of 10^{14} quanta/cm^2 corresponds to an absorption of 1 quantum per 100 chlorophyll molecules. Laser intensities were varied by a neutral density filter calibrated at 337 nm.


Delayed Light Emission Amplitude, arbitrary units
10
20
30
40
Incident Intensity, 10^{14} quanta/cm^2
0.2
0.4
0.6
0.8
1.0
Delayed light
$\Delta\phi_f$
0.5 ϕ_o
1.0 ϕ_o
1.5 ϕ_o
2.0 ϕ_o
$\Delta\phi_f$

prevent signal distortion by the anode circuit. A high current (2 ma) dynode chain was used and the photomultiplier anode current was maintained equal to or less than 5% of the dynode current to assure linear photomultiplier response. Transient fluctuations in dynode chain voltage were avoided by coupling of dynodes 8, 9 and 11 to ground with 0.1 μf disc capacitors.

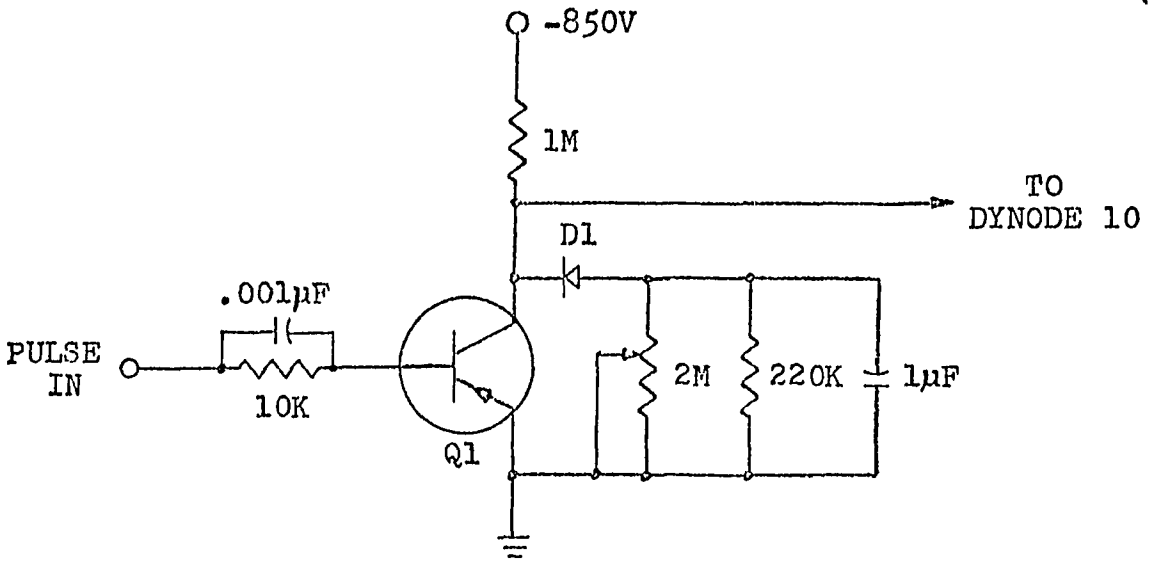
To avoid driving the photomultiplier into a nonlinear state during the intense fluorescence burst caused by the excitation flash, the photomultiplier was gated off electronically during excitation. Two gating methods were developed to reduce the photomultiplier gain during the excitation pulse. The first method was to control the bias voltage of dynode 10, the second dynode from the photomultiplier anode. Shifting dynode 10 to ground potential, using the circuit shown in Fig. 3 (a), caused the photomultiplier to be gated off (with a contrast ratio of 1000) in 1 μs for as long a period as desired and then gated on to normal gain within 2 μs. The second method was to control the bias voltage of dynode 1, the dynode adjacent the photomultiplier cathode. Shifting dynode 1 approximately 50 V more negative than the photocathode has been shown [41] to reduce photomultiplier gain. The circuit developed to control the first dynode bias is shown in Fig. 3 (b) and was also capable of gating the photomultiplier off within 1 μs and on to normal gain within 2 μs. The contrast ratio for this method was 500. Both methods of gating the photomultiplier were used in making delayed light emission measurements and were found to give identical results.

The delayed light emission decay was not recorded for times less than 6 μs since an artifactual signal, with an exponential decay time between

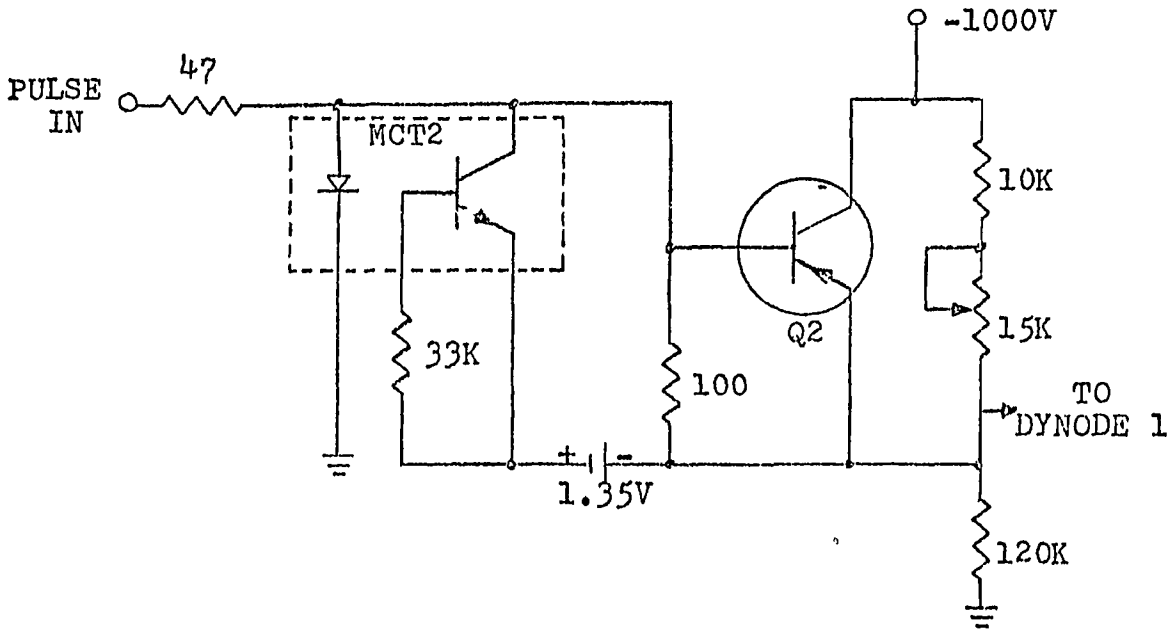
Fig. 3. Photomultiplier gating circuits.

Circuits used for gating the photomultiplier off during the actinic pulse. (a) This circuit was used to control the bias voltage to dynode number 10 of an EMI 9558B photomultiplier; D1 is a General Electric 1N4150 diode and Q1 is a Motorola MPS A92 transistor. (b) This circuit was used to control the bias voltage to dynode number 1 of an EMI 9558B photomultiplier; MCT2 is a Monsanto optical isolator and Q2 is a Motorola MPS A92 transistor. Resistances are given in ohms, kilo (K) or meg (M) ohms, voltage in volts (V) and capacitance in microfarads (μF).

(a)


-850V
1M
TO
DYNODE 10
D1
.001μF
PULSE
IN
10K
Q1
2M
220K
1μF

(b)

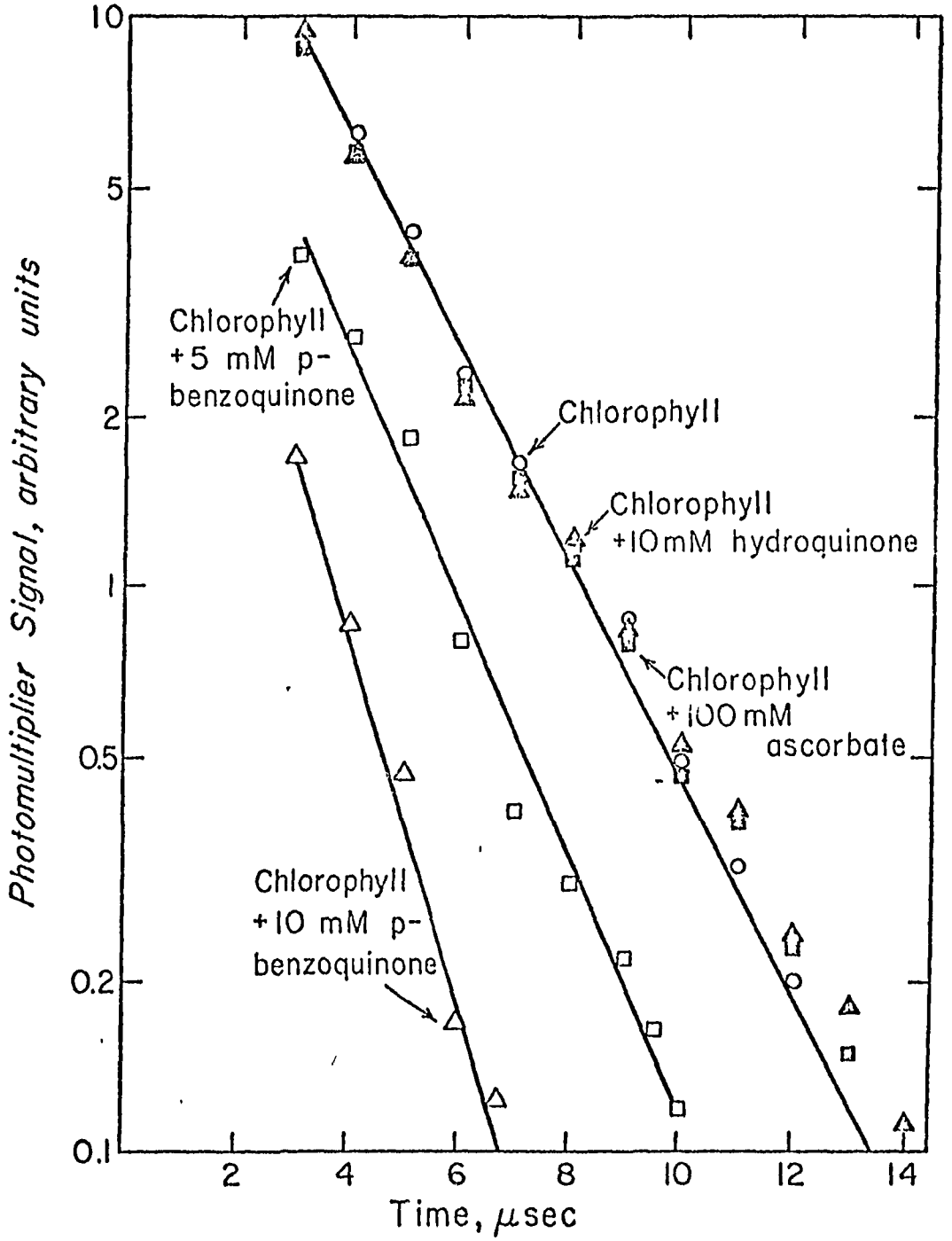

-1000V
47
PULSE
IN
MCT2
10K
Q2
15K
33K
100
TO
DYNODE 1
1.35V
120K

0.8-1.9 μs, was observed in this time range. It is believed this artifactual signal is generated by the intense burst of fluorescence from the excitation pulse, which causes a space charge to form in the photomultiplier even though it was shut off electronically during the excitation flash. This artifactual signal decayed away by 6 μs after this time. The generation of transient artifactual space charge signals in gated photomultipliers has been reported previously [42]. In order to determine what portion of the leading edge of the delayed light signal might be artifactual, a sample of chlorophyll a in solution, with fluorescence intensity identical to that of chloroplasts during the excitation flash, was used. The 0.8-1.9 μs signal from chlorophyll in solution was identical to a delayed light emission component with similar decay kinetics occurring at times less than 6 μs. Since chlorophyll in solution has been reported [43] to emit delayed light emission, it was possible that our 0.8-1.9 μs signal was not solely due to chlorophyll fluorescence. However, in the presence of ascorbate and hydroquinone, which only inhibit pigment delayed light emission [43], the 0.8-1.9 μs signal is not reduced (Fig. 4), but in the presence of p-benzoquinone, which also inhibits pigment fluorescence [43], it is reduced (Fig. 4). It was concluded that this 0.8-1.9 μs decaying signal was an artifact which decayed away by 6 μs allowing observation of true delayed light signal only at times greater than 6 μs.

Ultraviolet light from the laser was scattered by the sample and also generated an artifactual signal which was completely eliminated by the use of appropriate filters in front of the photomultiplier: a Kodak Wratten 2A and Schott RG-8. In making these measurements, the scattering properties of chloroplasts were approximated by a freshly prepared barium sulfate solution as described by Latimer [44].

Fig. 4. Photomultiplier signal produced from extracted chlorophyll a in solution.

Logarithmic plot of the decay of a photomultiplier signal generated from chlorophyll a in acetone-water (80%/20%, v/v) at a chlorophyll concentration of 0.25 μg/ml. The photomultiplier was gated off electronically during the flash and back on at 3 μs. Decays are shown for chlorophyll in solution (o—o—o), plus 10 mM hydroquinone (▲—▲—▲), plus 100 mM ascorbate (■—■—■), plus 5 mM p-benzoquinone (□—□—□), and plus 10 mM p-benzoquinone (Δ—Δ—Δ).


Photomultiplier Signal, arbitrary units
10
5
2
1
0.5
0.2
0.1
2
4
6
8
10
12
14
Time, μsec
Chlorophyll
+5 mM p-
benzoquinone
Chlorophyll
Chlorophyll
+10mM hydroquinone
Chlorophyll
+100 mM
ascorbate
Chlorophyll
+10 mM p-
benzoquinone

3. Signal Recording

Analog signals from the photomultiplier were digitized by a Biomation Model 805 transient waveform recorder. Signal averaging was accomplished by transfer of data from the waveform recorder to a Northern Scientific Model NA-514 digital averaging oscilloscope. Averaged data were printed out by teletype in numerical form for further analysis.

4. Timing Signals

All triggering signals for firing the laser, gating the photomultiplier and triggering the transient recorder were provided by the Tektronix type 160 series pulse and waveform generators. The photomultiplier was gated off 2 μs before the laser was fired and was turned on by 6 μs after the laser flash. The transient recorder was triggered at the same time the laser was fired to establish the time base zero point.

D. Chlorophyll a Fluorescence Yield Measurements after a Flash

The chlorophyll a fluorescence yield (ϕ_f) rise after an excitation flash was measured by a method similar to that of Mauzerall [45]. A weak analytic flash, which itself did not cause a change in fluorescence yield, was used to generate fluorescence signals proportional to the yield of fluorescence at various time delays beginning 3 μs after the excitation flash. It was possible to begin measuring 3 μs versus 6 μs for delayed light emission, since the fluorescence signal, even at 3 μs, was large enought to be easily distinguished from the artifactual signal. The analytical flash was provided by a General Radio Model 1538-A strobe lamp through neutral density filters, two Corning CS 4-96 glass filters and a light guide (Fig. 1). The fluorescence yield was calculated at a particular time after the main actinic flash using the following formula:

$$\phi_f(t) \quad \text{(in terms of } \phi_0\text{)} = \frac{F_m(t) - I_{DLE}(t)}{F_0}$$

where $\phi_f(t)$ is the fluorescence yield at time t after the main actinic pulse $F_m(t)$ is the measured fluorescence signal at some time t, $I_{DLE}(t)$ is the intensity of delayed light emission at some time t, F_0 is the zero level fluorescence signal generated by pulsing the weak analytic flash without the main actinic flash, and ϕ_0 is the fluorescence yield prior to an actinic flash. ϕ_o is determined in samples dark adapted for 5 min or longer since after an excitation flash ϕ_f decays to the ϕ_o level in about 5 min.

E. Oxygen Measurements with Continuous Light

The oxygen evolving rate of chloroplasts under continuous illumination was measured with a Yellow Springs Instrument platimum/Ag-$AgCl_2$ Clark electrode and Model 53 oxygen monitoring system. Chloroplasts were normally at a chlorophyll concentration of 50 μg/ml with 0.1 mM ferricynanide as an electron acceptor. Saturating continuous illumination (250 mW/cm^2) was provided by an incandescent lamp through a Corning CS 3-71 glass filter and a two inch water filter.

F. Oxygen Measurements with Flashing Light

Oxygen evolution under flashing light conditions (O_2 per flash as a function of flash number in a series of flashes) was measured on a Joliot type electrode [46] using a General Radio Model 1538-A strobe lamp for excitation. Saturating flashes were given at a rate of one flash per second and data was recorded with a Midwestern Instruments Model 801 oscillograph.

CHAPTER III

THE EFFECT ON TRIS WASHING ON DELAYED LIGHT EMISSION AND THE RISE IN CHLOROPHYLL A FLUORESCENCE YIELD

A. Introduction

The reaction center complex of Photosystem II uses the excited singlet state energy of chlorophyll a molecule to generate a charge couple. This reaction is believed to take place in the following schematic form [1, 12]:

$$ZP_{680}Q \xrightarrow{h\nu} ZP^{*}_{680}Q \longrightarrow ZP^{+}_{680}Q^{-}$$

where Z is the first secondary electron donor, P_{680} is the primary electron donor and the reaction center chlorophyll a shown in its ground state, singlet excited state (*) and oxidized form (+) and Q is the first "stable" primary electron acceptor. It has been hypothesized that microsecond delayed light emission is generated by the back reaction of P^{+}_{680} with Q^{-} [20, 24].

Delayed light emission is, of course, a misuse of the photo-generated Photosystem II redox energy. Large scale loss of energy by this means is prevented by a stabilization step, the rapid reduction of P^{+}_{680} by Z. Thus, it is expected that delayed light emission, especially in the microsecond time range, will be sensitive to alterations in the stabilization of the Photosystem II redox energy.

A number of investigations have established a correlation between the charge accumulation on the donor side of Photosystem II and the amplitude of various components of delayed light emission [17]. Hydroxylamine, which

is believed to inhibit the $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ reaction, increases the amplitude of a delayed light component with a half time ($\tau_{\frac{1}{2}}$) of 35 μs [47], while conditions designed to maximize the concentration of Z^{+} stimulate a component with $\tau_{\frac{1}{2}}$ of 120 μs [48]. The rise in chlorophyll a fluorescence yield in the 0-20 μs time range, after an excitation flash, is indicative of the variations in P^{+}_{680} concentration, since P^{+}_{680} is a quencher of fluorescence [14, 15]. A low fluorescence yield at 16 μs after a flash correspond with an increased amplitude of a 20 μs component of delayed light emission [49].

If the above ideas of the origin of microsecond delayed light emission are correct, then one expects that any treatment which alters the coupling between Z and the charge accumulating oxygen evolving system will simultaneously affect P^{+}_{680} and delayed light emission. Various treatments which modify this electron transfer reaction may be used for this purpose, such as heating [50], incubation with chaotropic agents [51], Alkaline Tris (hydroxymethyl)aminomethane (TRIS) washing [52], and hydroxylamine treatment [53, 54]. TRIS treatment was used here because its effect on the behavior of Photosystem II reactions have been well characterized by electron spin resonance (ESR) and polarographic techniques [55]. Furthermore, addition of exogenous electron donors ($MnCl_2$, ascorbate, phenylenediamine and benzidine) to TRIS washed chloroplasts has been used to alter Photosystem II reactions.

Both the delayed light emission decay and the chlorophyll a fluorescence yield rise were measured in the microsecond time range after a saturating flash. Since the fluorescence yield rise is related to the concentration of P^{+}_{680} [15, 56] its measurement enabled us to monitor how various treat-

ments affected P_{680}^{+}; this information was essential for interpreting delayed light emission. The basic experimental method and hypotheses used here are similar to those used by Duysens et al. [49] for the intact algal cells. However, previous authors have not measured both delayed light emission and chlorophyll a fluorescence yield in the same sample. Variations of Photosystem II reactions combined with the measurements of both fluorescence yield rise and delayed light emission, after a saturating 10 ns actinic flash, have now provided experimental evidence for the $P_{680}^{+}Q^{-}$ recombination hypothesis for microsecond delayed light emission from isolated chloroplasts.

B. Materials and Methods

In these experiments chloroplasts from Alaska pea and bush bean leaves were used. The plant material was grown in our laboratory; procedure for chloroplast extraction is given in Chapter II. TRIS washing of chloroplasts was carried out as described by Blankenship and Sauer [57] in 0.8 M TRIS, pH = 8.0, at 0 ^{o}C for approximately 20 min. The effectiveness of TRIS washing was tested by measuring the rate of oxygen evolution under saturating continuous illumination using ferricyanide as an electron acceptor. A Yellow Springs Instrument Clark Electrode and Model 53 Oxygen Monitoring System were used for these measurements. All TRIS washed chloroplast samples, used in these experiments, showed at least 90% inhibition of oxygen evolution. Samples were also tested on a Joliot type electrode [46] to determine their oxygen evolution capacity under flash illumination conditions.

The apparatus and protocol used in measuring delayed light emission decay and chlorophyll a fluorescence yield rise have been described in Chapter II and in ref. 58.

The delayed light decay data for samples of various treatments were always compared to an untreated control sample of a particular experimental run. This was necessary since the amplitudes and decay times of the delayed light emission components varied by approximately ten percent from one preparation to another. Since the delayed light emission decays are complex functions, they were plotted in semilogarithmic form and standard graphical procedures were used to calculate amplitudes and lifetimes of exponential components. The resultant components may not have any particular significance by themselves but provide a means of comparing the changes in delayed light emission decay with various treatments. The mechanistic reality of a delayed light component can only be demonstrated by correlation with other measurements.

C. Results

1. TRIS Washed Chloroplasts with Various Modes of Excitation

The effect of TRIS washing on the delayed light emission decay and the fluorescence yield rise after single flash excitation and after the final flash following different preillumination conditions was investigated. Delayed light emission decays after the final flash of a series of flashes given every 0.5 s are shown in Fig. 5. The component lifetimes and amplitudes resolved from these semilogarithmic plots are shown in Table 1, lines 1 and 2. TRIS washing had a significant effect both on the 6 μs and 30 μs components. TRIS washing decreases the amplitude of the 6 μs component but does not change its lifetime. The 30 μs component is eliminated, and a component with a lifetime of 60-70 μs becomes predominant. Under the same experimental conditions TRIS washing eliminated almost all of the rise in fluorescence yield as can be seen in Fig. 6.

Fig. 5. Delayed light emission decay in TRIS washed chloroplasts.

Logarithmic plot of delayed light emission decay from 6 to 100 μs after the final flash of a series of flashes given at a rate of 2 flashes/s. Bush bean chloroplasts: control (o—o—o) and TRIS washed (△—△—△).

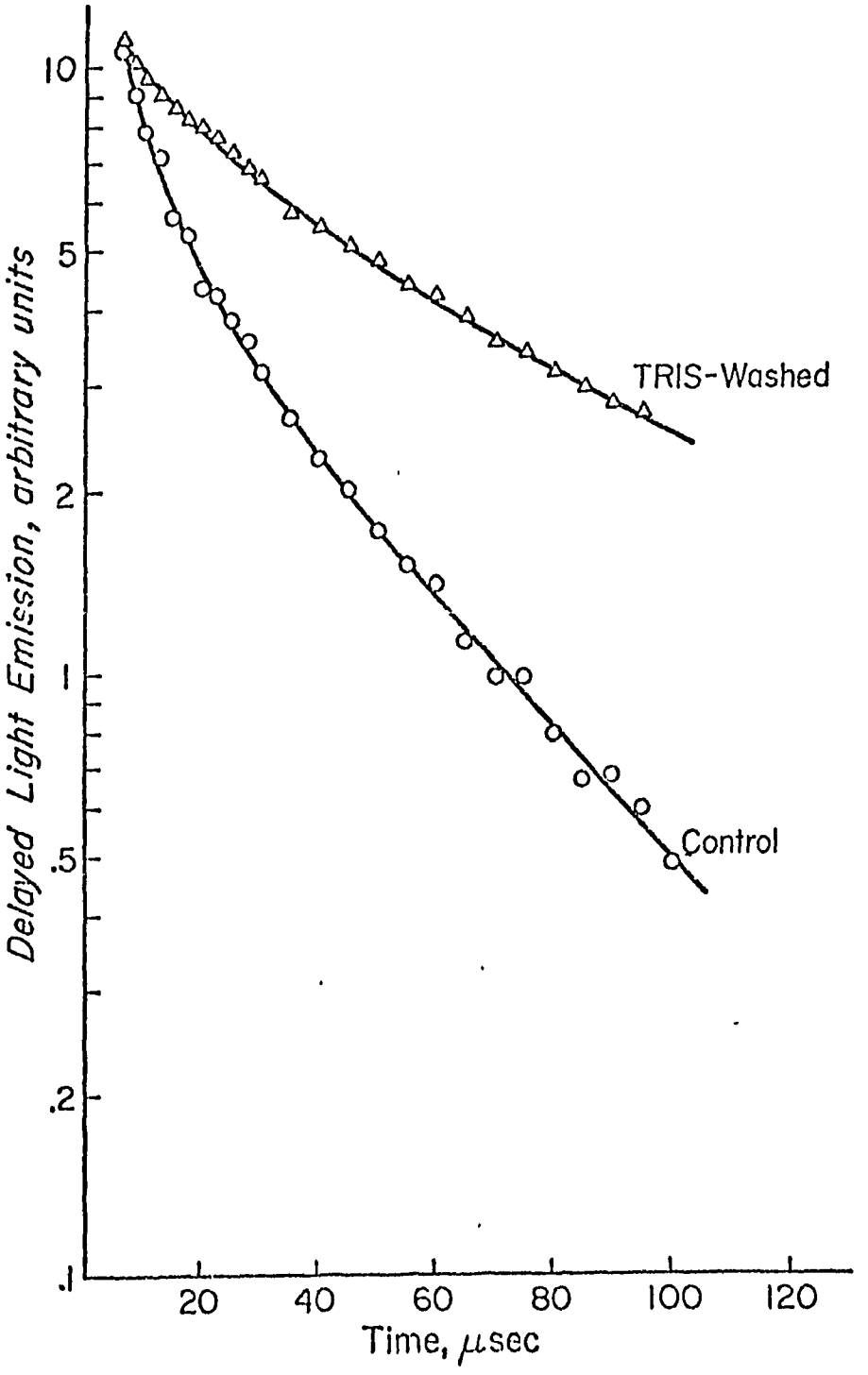

Delayed Light Emission, arbitrary units
10
5
2
1
.5
.2
.1
20
40
60
80
100
120
Time, μsec
TRIS-Washed
Control

TABLE 1.

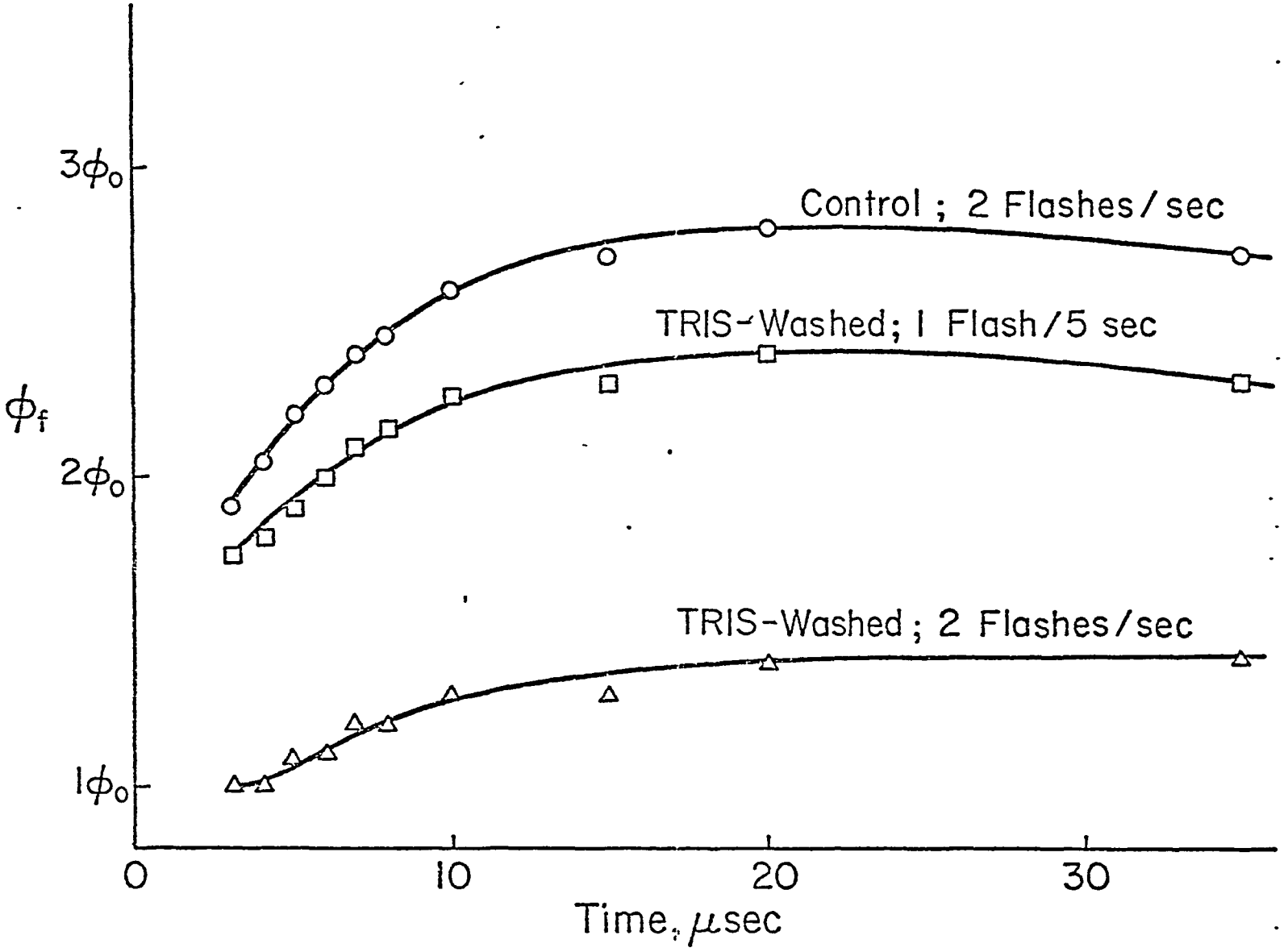
Decay Characteristics of Delayed Light Emission in the 6-100 μs Range for TRIS Washed Samples

Standard graphical procedures were applied to semilog plots of delayed light emission decays to calculate amplitudes (α) and lifetimes (τ). Amplitude values are given in percentages of the extrapolated zero time value for delayed light emission $\Sigma\alpha = 100\%$ and τ values are given in microseconds. Typical measurement errors given as one standard deviation unit are indicated. The area under the curve was calculated using the following formula: Area = $\sum_i \alpha_i \tau_i$ and was normalized to one for the appropriate control.

Line	Sample Conditions; Bush Bean Chloroplasts	α_1	$\tau_{1,\ \mu s}$	α_2	$\tau_{2,\ \mu s}$	Area
1	Control, 1 flash/0.5 s	59 ± 6	6 ± 0.8	41 ± 6	32 ± 4	1.0
2	Tris washed, 1 flash/0.5 s	34	7	66	70	2.2
3	Tris washed, 1 flash/5 s	61	6	39	39	1.1

Fig. 6. Chlorophyll a fluorescence yield rise in TRIS washed chloroplasts.

Plot of the rise in chlorophyll a fluorescence yield (ϕ_f in terms of ϕ_0, the level of fluorescence yield prior to excitation) after the final flash of a series of flashes. Bush bean chloroplasts; excitation rate, 2 flashes/s: control (o—o—o) and TRIS washed (△—△—△); excitation rate, 1 flash/5s: TRIS washed (□—□—□).


3φo
2φo
1φo
φf
Control ; 2 Flashes / sec
TRIS-Washed; 1 Flash / 5 sec
TRIS-Washed ; 2 Flashes / sec
0
10
20
30
Time, μsec

Delayed light emission decay and fluorescence yield measurements were also made under other excitation conditions. In control samples that showed strong period of four oscillations in oxygen yield past the thirteenth flash, the delayed light emission amplitude at 90 μs also oscillated with flash number. These oscillations in amplitude were smaller than has been previously reported [49, 59] and damped out by the ninth flash. The delayed light components at $\tau<30$ μs were found not to oscillate with period four but to be low on the first flash, high on the second, and remain constant thereafter.

TRIS treated chloroplasts were not expected to be sensitive to flash number since their Photosystem II reaction centers are disconnected from the oxygen evolving system. It was observed that the first flash after a 10 min dark adaptation period gave a delayed light emission decay very similar to that of the control sample. The second flash gave a delayed light emission decay intermediate to that of the control and the TRIS washed sample receiving many flashes. The third and all succeeding flashes, given with a period of 1 s or less, gave the delayed light emission decay identified as the TRIS washed sample in Fig. 5. With excitation flashes given every 5 s the delayed light emission decay after the final flash approached that of the control sample (compare lines 1 and 3 of Table 1). Excitation flash periods between 0.5 and 5 s gave delayed light emission decays with amplitudes and halftimes intermediate to that shown in lines 2 and 3 of Table 1.

In control chloroplasts the delayed light emission decay and flurorescence yield rise, after the final flash in a long series of flashes, were essentially the same if the flashes were given at a rate of 1 flash/s or 20 flashes/s.

In TRIS washed samples, the fluorescence yield rise observed after the first flash after dark adaptation was essentially the same as that of the control sample in Fig. 6. When the fluorescence yield rise is plotted as an exponential, the rise time of the control is approximately 6 μs. It should be noted that if one plots log $(\phi_f(t) - \phi_0)$ versus time, then a straight line is found. Thus, the chlorophyll a fluorescence yield rises exponentially according to the following equation:

$$\phi_f(t) - \phi_0 = [\phi_f(\max) - \phi_0]\ [1 - e^{-t/\tau}]$$

where ϕ_0 is the level of fluorescence yield prior to a saturating excitation flash, $\phi_f(t)$ is the fluorescence yield in terms of ϕ_0 at a time t after a saturating excitation flash, $\phi_f(\max)$ is the maximum level attained by $\phi_f(t)$, and τ is the exponental rise lifetime. The flash repetition rate also affected the fluorescence yield rise. A flashing rate of 1 flash/5 s gave a fluorescence yield rise in a TRIS washed sample that approached control; see Fig. 6. Excitation flash rates with periods between 0.5 and 5 s gave the fluorescence yield rises intermediate to those shown in Fig. 6.

2. TRIS Washed Chloroplasts with Various Electron Donors

a. Mn^{2+} Donation

According to interpretations of electron spin resonance data [55] Mn^{2+} acts as an effective donor of electrons to P^+_{680} in TRIS washed chloroplasts. The effect of Mn^{2+} on delayed light emission decay, following the final flash of a series given with a period of 1 s, is shown in Fig. 7 and Table 2. The addition of 10^{-7} M $MnCl_2$ ($MnCl_2$:Chlorophyll = 1:50) reverses the changes in delayed light emission decay caused by TRIS

Fig. 7. Delayed light emission decay in TRIS washed chloroplasts plus $MnCl_2$.

Logarithmic plot of delayed light emission decay from 6 to 100 μs after the final flash in a series of flashes given at a rate of 1 flash/s. Alaska pea chloroplasts; control (o—o—o), TRIS washed (□—□—□), TRIS washed plus 10^{-7} M $MnCl_2$ (x—x—x), and TRIS washed plus 10^{-7} M $MnCl_2$ and 10^{-5} M EDTA (△—△—△).

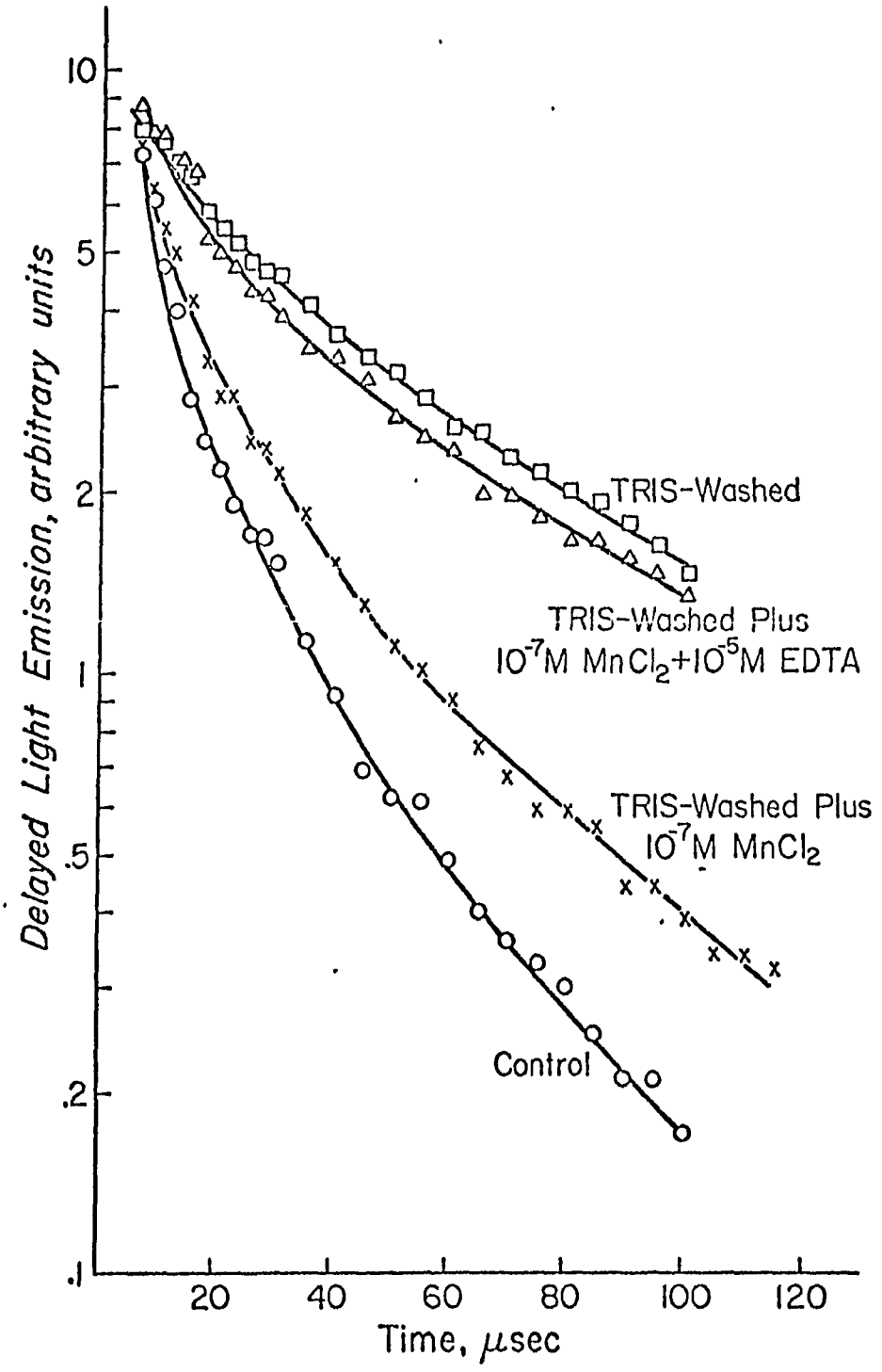

Delayed Light Emission, arbitrary units
10
5
2
1
.5
.2
.1
20
40
60
80
100
120
Time, μsec
TRIS-Washed
TRIS-Washed Plus
10⁻⁷M MnCl₂+10⁻⁵M EDTA
TRIS-Washed Plus
10⁻⁷M MnCl₂
Control

TABLE 2.

Decay Characteristics of Delayed Light Emission in the 6-100 μs Range for TRIS Washed Samples Plus $MnCl_2$, Plus Ascorbate and Ascorbate with Phenylenediamine. Other information as in Table 1.

Line	Sample Conditions; Alaska pea Chloroplasts	α_1	τ_1, μs	α_2	τ_2, μs	Area
1	Control, 1 flash/s	73	7	27	32	1.0
2	TRIS washed, 1 flash/s	39 ± 7	6 ± 0.8	61 ± 7	59 ± 6	2.3 ± 0.3
3	TRIS washed, 10^{-7}M $MnCl_2$, 1 flash/s	70	7	30	51	1.4
4	TRIS washed, 10^{-7}M $MnCl_2$, 10^{-5}M EDTA, 1 flash/s	47	7	53	58	2.4
5	TRIS washed, 10^{-4}M ascorbate, 1 flash/s	64	6	36	35	0.97
6	TRIS washed, 20 flash/s	40	7	60	60	2.6
7	TRIS washed, 10^{-4}M Ascorbate, 20 flash/s	48	7	52	58	2.2
8	TRIS washed, 10^{-4}M Ascorbate, 10^{-5}M Phenylenediamine, 20 flashes/s	75	7	25	32	1.2

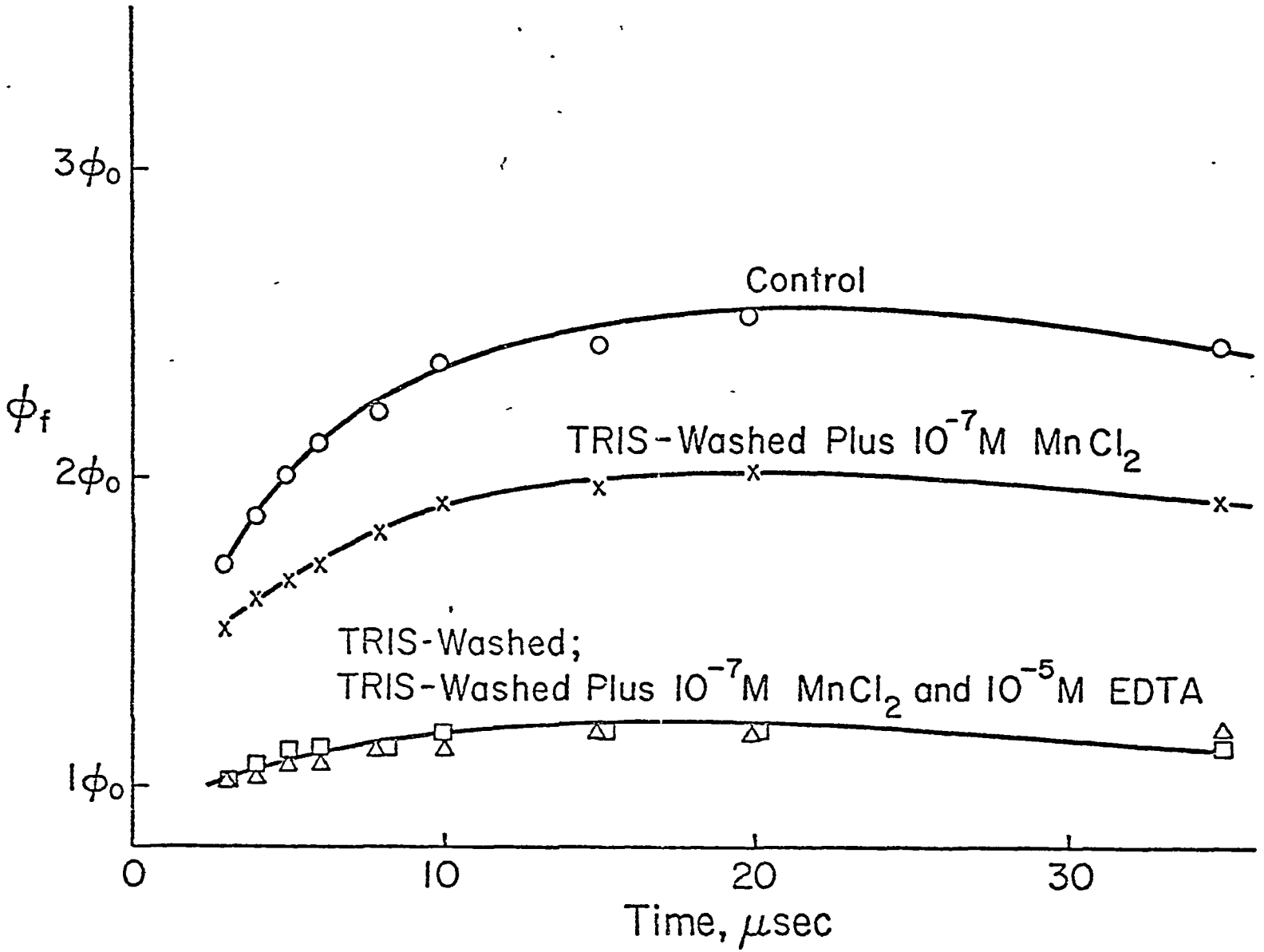
washing. It is important to note that Mn^{2+} is peculiar since it causes the amplitude (α) of the slower component to approach that of the control while the lifetime (τ) remains unchanged from that in the TRIS-washed sample (compared lines 1-3 of Table 2). It is believed (see later) this reflects Mn^{2+} donating electrons to P^{+}_{680} via Z. With the addition of 10^{-5} M ethylenediamine tetracetic acid (EDTA), a chelator of Mn^{2+}, both the amplitudes and decay halftimes reverted almost fully to that of a TRIS treated sample with no additions (Fig. 7). A further reversal of the Mn^{2+} effect was not observed with greater concentrations of EDTA. The effects on the individual decay components are shown in Table 2, lines 2-4. When higher concentration of $MnCl_2$ (1 $MnCl_2$ per 5 chlorophyll molecules, as in ref. 55) was used, the same changes in the delayed light emission decay was observed as with lower concentrations. TRIS treated samples have Mn released to the inside of the thylakoid [55], thus, the ability of $MnCl_2$ at an external concentration of 10^{-7}M to act as a donor suggests the site of donation is on the outside of the thylakoid not the inside.

Measurements of the rise in fluorescence yield after the final excitation flash under these conditions are shown in Fig. 8. As in the delayed light emission case, addition of $MnCl_2$ causes the rise in fluorescence yield in the TRIS washed chloroplasts to approach that of the control. The subsequent addition of EDTA also reversed the Mn^{2+} effect on the fluorescence yield in TRIS washed chloroplasts.

The observation that the TRIS effect on delayed light emission and fluorescence yield is maximum on the third flash is interpreted (see discussion) to mean that two charge carriers must exist between the site of TRIS blockage and P_{680} and these charge carriers must be oxidized to see the

Fig. 8. Chlorophyll a fluorescence yield rise in TRIS washed chloroplasts plus $MnCl_2$.

Plot of the rise in chlorophyll a fluorescence yield after a final flash in a series of flashes. Alaska pea chloroplasts; control (o—o—o), TRIS washed (□—□—□), TRIS washed plus 10^{-7} M $MnCl_2$ (x—x—x), and TRIS washed plus 10^{-7} M $MnCl_2$ and 10^{-5} M EDTA (△—△—△).


3ϕo
2ϕo
1ϕo
ϕf
Control
TRIS-Washed Plus 10^-7 M MnCl2
TRIS-Washed;
TRIS-Washed Plus 10^-7 M MnCl2 and 10^-5 M EDTA
0
10
20
30
Time, μsec

TRIS effect. Addition of 10 mM ferricyanide to TRIS-washed chloroplasts, during 10 min dark adaptation, did not change the TRIS effect suggesting that this chemical was unable to oxidize these charge carriers.

b. Ascorbate and Ascorbate Plus Phenylenediamine

In TRIS washed chloroplasts, ascorbate and ascorbate plus phenylenediamine were also used as electron donors to Photosystem II. Comparison of lines 1, 2 and 5 in Table 2 shows that addition of 10^{-4} M ascorbate to TRIS washed chloroplasts gave rise to a delayed light emission decay very similar to the control for a flash period of 1 s. However, at a flash rate of 20 flashes/s the addition of ascorbate to TRIS washed chloroplasts was ineffective (lines 1, 6 and 7 of Table 2). Addition of 10^{-5} M phenylenediamine and 10^{-4} M ascorbate gave decay kinetics similar to that of the control even with excitation flash rates of 20 flashes/s (see lines 1, 2 and 8 of Table 2).

c. Ascorbate Plus Benzidine

A different system for donating electrons to Photosystem II in TRIS washed chloroplasts was desired in order to test the generality of the changes produced by electron donors on delayed light emission decay and fluroescence yield rise. Ascorbate plus benzidine was chosen since there are ESR data which show how this electron donating system affects Photosystem II in TRIS washed chloroplasts [55].

Ascorbate plus benzidine caused the amplitudes of the 6 μs component to approach that of the control, while the amplitude and lifetime of the slower component were also reduced to those of the control (see lines 1-3 of Table 3 and Fig. 9). It should be noted that this occurred even with high flash repetition rates (20 flashes/s).

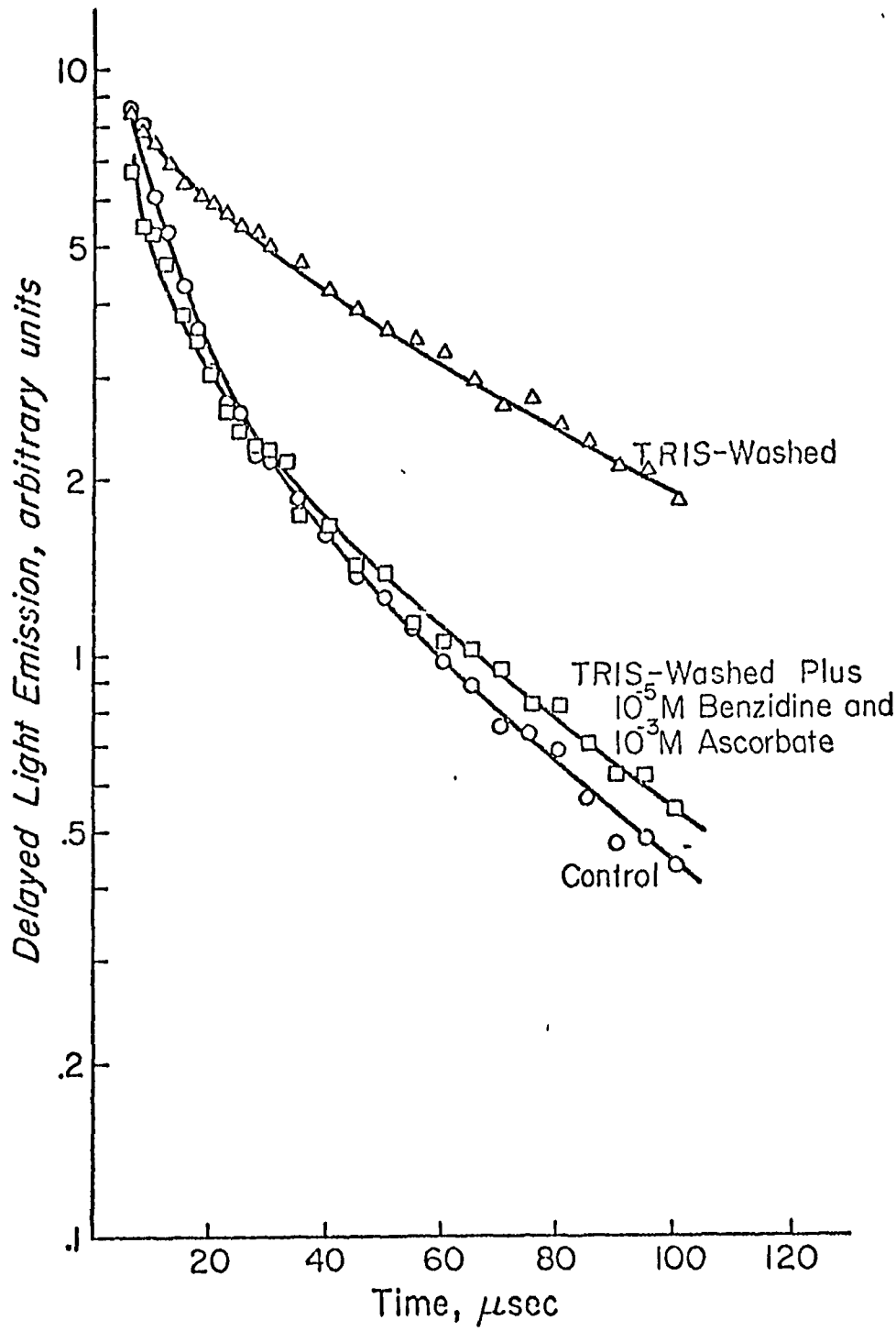
TABLE 3.

Decay Characteristics of Delayed Light Emission in the 6-100 μs Range for TRIS Washed Samples Plus Benzidine with Ascorbate. Other information as in the legend of Table 1.

Line	Sample Conditions	α_1	τ_1, μs	α_2	τ_2, μs	Area
1	Control, 1 flash/s	73	6	27	36	1.0
2	TRIS washed, 1 flash/s	35 ± 7	6 ± 0.8	65 ± 7	70 ± 7	2.2 ± 0.3
3	TRIS washed, 10^{-3}M Ascorbate, 10^{-5}M Benzidine 20 flashes/s	70	6	30	42	0.87

Fig. 9. Delayed light emission decay in TRIS washed chloroplasts plus ascorbate and benzidine.

Logarithmic plot of the decay in delayed light emission after a final flash following a series of preillumination flashes: untreated control chloroplasts with preillumination flashes at a rate of 1 flash/s (o—o—o), TRIS washed chloroplasts, 1 flash/s (△—△—△), and TRIS washed chloroplasts with 10^{-3} M ascorbate plus 10^{-5} M benzidine, 20 flashes/s (□—□—□).


Delayed Light Emission, arbitrary units
10
5
2
1
.5
.2
.1
20
40
60
80
100
120
Time, μsec
TRIS-Washed
TRIS-Washed Plus
10⁻⁵M Benzidine and
10⁻³M Ascorbate
Control

The effect of ascorbate and benzidine on the rise in fluorescence yield of TRIS washed chloroplasts is shown in Fig. 10. At a flash rate of 20 flashes/s the TRIS washed sample with ascorbate and benzidine had a slightly larger variable yield of fluorescence than the control.

3. Area Under the Curve

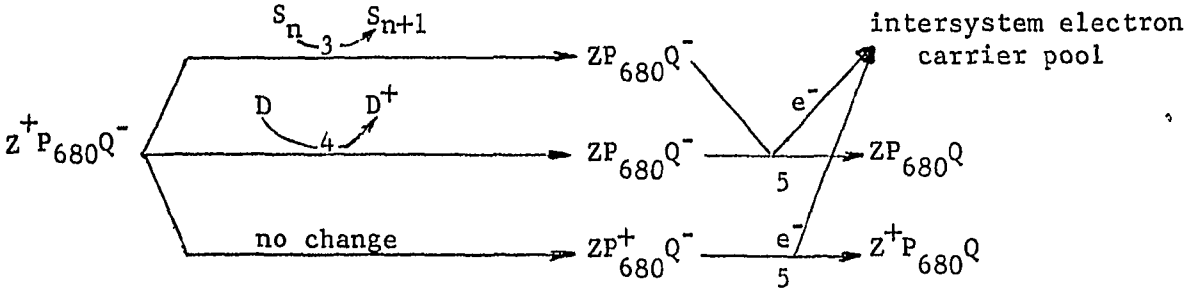
The area under the delayed light decay curve due to the components observed is shown in Tables 1-3. The area is representative of the amount of charge recombination that is occurring in this time range. As expected for TRIS washed samples, where the charge stabilization reactions have been inhibited, the area under the delayed light decay curves is two to three times greater than for the control.

D. Discussion

In this discussion the following diagram of the primary processes believed to be occurring at the Photosystem II reaction center will be used:

$$ZP_{680}Q \underset{h\nu_{DLE}}{\overset{h\nu}{\rightleftharpoons}} ZP^{*}_{680}Q \underset{1}{\overset{\tau_{1/2} < 20\ ns}{\rightleftharpoons}} ZP^{+}_{680}Q^{-} \underset{2}{\overset{\tau = 6\ \mu s}{\rightleftharpoons}} Z^{+}P_{680}Q^{-}$$

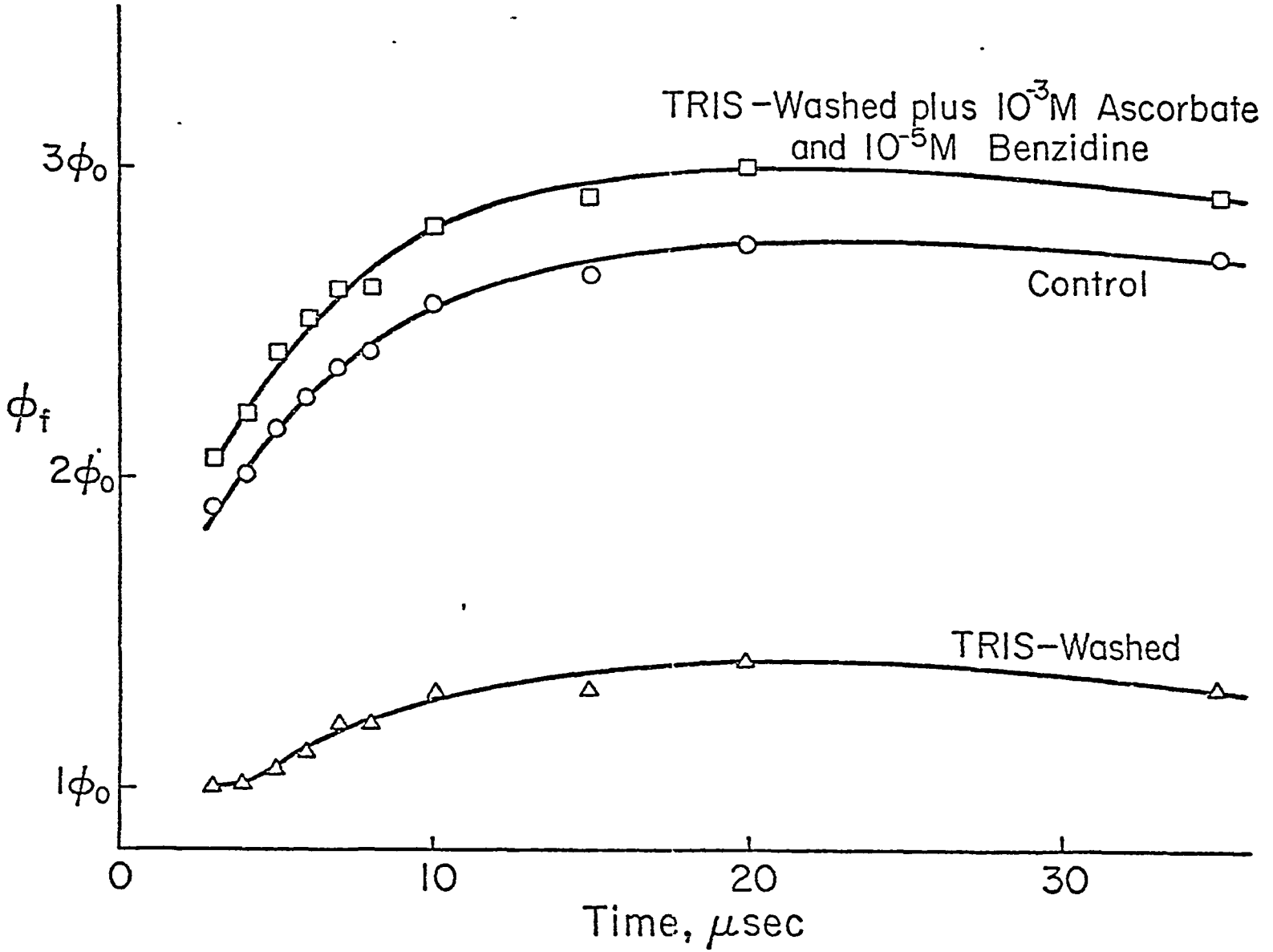
(ϕ_f low) (ϕ_f high)



where Z and Q are the first secondary electron donor and the first "stable" acceptor for Photosystem II, P^{+}_{680} is the primary electron donor and the

Fig. 10. Chlorophyll a fluorescence yield rise in TRIS washed chloroplasts plus ascorbate and benzidine.

Plot of the rise in chlorophyll a fluorescence yield (ϕ_f) in terms of ϕ_0, the level of fluorescence yield prior to excitation. Untreated control chloroplasts (o—o—o), TRIS washed chloroplasts (△—△—△), and TRIS washed chloroplasts with 10^{-3} M ascorbate plus 10^{-5} M benzidine (□—□—□).


TRIS–Washed plus 10^{-3}M Ascorbate
and 10^{-5}M Benzidine
Control
TRIS–Washed
$3\phi_o$
$2\phi_o$
$1\phi_o$
ϕ_f
0
10
20
30
Time, μsec

reaction II chlorophyll *a*, S_n and S_{n+1} represent charge accumulating sequential states of the oxygen evolving system, D is a secondary electron donor, and ϕ_f stands for quantum yield of fluorescence. Reaction 1 (left to right) is the formation of the primary redox couple from the excited (*) singlet reaction center chlorophyll energy. Reaction 2 (left to right) is the electron transfer reaction from Z to P_{680}^+ which is believed to be a stabilization step that curtails delayed light emission. Its lifetime of 6 μs is obtained from fluorescence rise curves such as in Fig. 6. Reaction 3 is the movement of electrons from the oxygen evolving system to Z^+. This reaction is inhibited by TRIS washing. Reaction 4 is the reduction of Z^+ by a donor (D) of electrons. Reactions 5 represent the movement of electrons from Q^- to the intersystem electron transport chain. Reactions 1 and 2 are reversible and give rise to the singlet excited state of the reaction center chlorophyll and thus delayed light emission ($h\nu_{DLE}$).

ESR data has suggested that TRIS washing and the treatment with various electron donors affect the formation and the rate of decay of Z^+ [55]. Whether or not TRIS washing has an effect on reaction 2 of the above scheme could not be determined from the ESR studies due to a 100 μs response time limitation. The rise in fluorescence yield, however, is believed to be directly related to the reduction of P_{680}^+ [15, 49, 58]. The present results show that TRIS washed samples can exhibit the same fluorescence rise kinetics as untreated ones provided they are sufficiently dark adapted or receive a flash repetition rate of $\leq$ 0.2 Hz. Thus, it appears that TRIS washing has no effect on the movement of electrons from Z to P_{680}^+ (reaction 2).

The concentration of P_{680}^+ in the Photosystem II reaction centers can be controlled by the use of various electron donors and preillumination

routines. A low concentration of P^+_{680} results in a large fluorescence yield and a low amplitude of rapidly decaying delayed light emission, whereas a high concentration of P^+_{680} gives only a low fluorescence yield, and a large amplitude of slowly decaying delayed light emission. The effect of TRIS washing on the rise in fluorescence yield can be explained by a block in reaction 3, but reduction of Z^+ still occurs with a halftime of 1 s or greater by reaction 4 and charge recombination. ESR measurements have established representative halflifes for Z^+ reduction under various experiment conditions [55]. With a flash repetition rate of $\geq$1 Hz $Z^+P^+_{680}Q^-$ rapidly builds up and fluorescence is quenched. However, as can be seen in Fig. 6, even though a large portion of the rise in fluorescence yield has been eliminated, a small amount remains. This would reflect the partial regeneration, between excitation flashes, of $ZP_{680}Q$ by reactions 4 and 5.

When exogenous donors are added the halftime of reaction 4 can be greatly accelerated and $Z^+P^+_{680}Q^-$ will not build up. Thus $MnCl_2$, ascorbate, ascorbate plus phenylenediamine and ascorbate plus benzidine reverse the effect of TRIS washing on the rise in fluorescence yield (Figs. 8 and 10). The combination of ascorbate plus benzidine as electron donor actually enhanced the fluorescence yield rise relative to the control. This may indicate a greater efficiency of electron donation than the endogenous donor.

Reversal of the TRIS effect by a donor is dependent on the rate of donation relative to flash repetitive rate. Thus 10^{-4} M ascorbate will have an electron donation time [55] which will make it of limited effectiveness at repetition rates greater than about 2 or 3 Hz. Indeed this is shown to be the case for samples with ascorbate receiving flash excitation at a rate of 20 Hz (Table 2 line 6).

The observed low fluorescence yield in TRIS washed chloroplasts given continuous illumination [52, 60, 61] can now be explained in a manner consistent with the flash excitation results presented here. Under continuous illumination reaction 4, having a halftime of greater than 1 s, is unable to regenerate $ZP_{680}Q$. As a result $Z^+P^+_{680}Q$ and $Z^+P^+_{680}Q^-$ build up and act as quenchers of fluorescence.

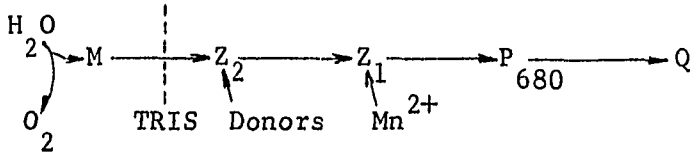
Delayed light emission in the time range $6<t<100$ μs is observed to be enhanced by TRIS washing. As explained for the rise in fluorescence yield, TRIS washing will result in a build up of $Z^+P^+_{680}Q^-$ which will augment recombination giving rise to more intense delayed light emission (see Fig. 5 and line 1 and 2 of Table 1). Flash excitation given with a period of 5 s, greater than the 1 s halftime of reaction 4 in TRIS washed chloroplasts, resulted in delayed light emission close to that of the control (see line 3 of Table 1). This is expected since during the 5 s between flashes $Z^+P_{680}Q^-$ will be dissipated by reaction 4 and charge recombination.

With the addition of electron donors which lower the halftime of reaction 4 the enhancement of delayed light emission by TRIS washing is abolished (see Fig. 9, lines 5 and 8 of Table 2, and line 3 of Table 3). The addition of $MnCl_2$ as an electron donor is different than other electron donors since it inhibits the TRIS washing enhancement of delayed light emission while not lowering the halftime of reaction 4 (see line 3 of Table 2). Also, in TRIS washed chloroplasts with Mn^{2+} as an electron donor, the rise in fluorescence yield approaches that of control with a 6 μs rise lifetime. This suggests that Mn^{2+} donates electrons to P^+_{680} through Z i.e., the Mn^{2+} donation rate is determined by reaction 2 and like control has a lifetime of 6 μs.

The most rapidly decaying component of delayed light emission, beginning at 6 μs after the flash, has a decay time of 6 μs and the fluorescence yield rise, beginning at 3 μs after the flash, has a rise time of 6 μs (Fig. 11). TRIS washing decreases the 6 μs delayed light and the fluorescence rise components (Fig. 11(b)). Mn^{2+} and other electron donors reverse this TRIS effect. The same 6 μs lifetime and the correlation of TRIS and donor effects on both delayed light emission decay and fluorescence yield rise, we believe, indicates that the 6 μs delay light emission decay reflects reaction 2, a charge stabilization reaction.

Our data indicate that the decrease observed in the variable yield of fluorescence (indicating a higher percentage of centers in the $P^{+}_{680}Q^{-}$ form) is greater than the change in the amplitude of the 6 μs delayed light emission component. This indicates that a strict quantitative relationship between the decrease in fluorescence yield (increase in percentage of $P^{+}_{680}Q^{-}$ centers) and the amplitude of delayed light emission is not possible.

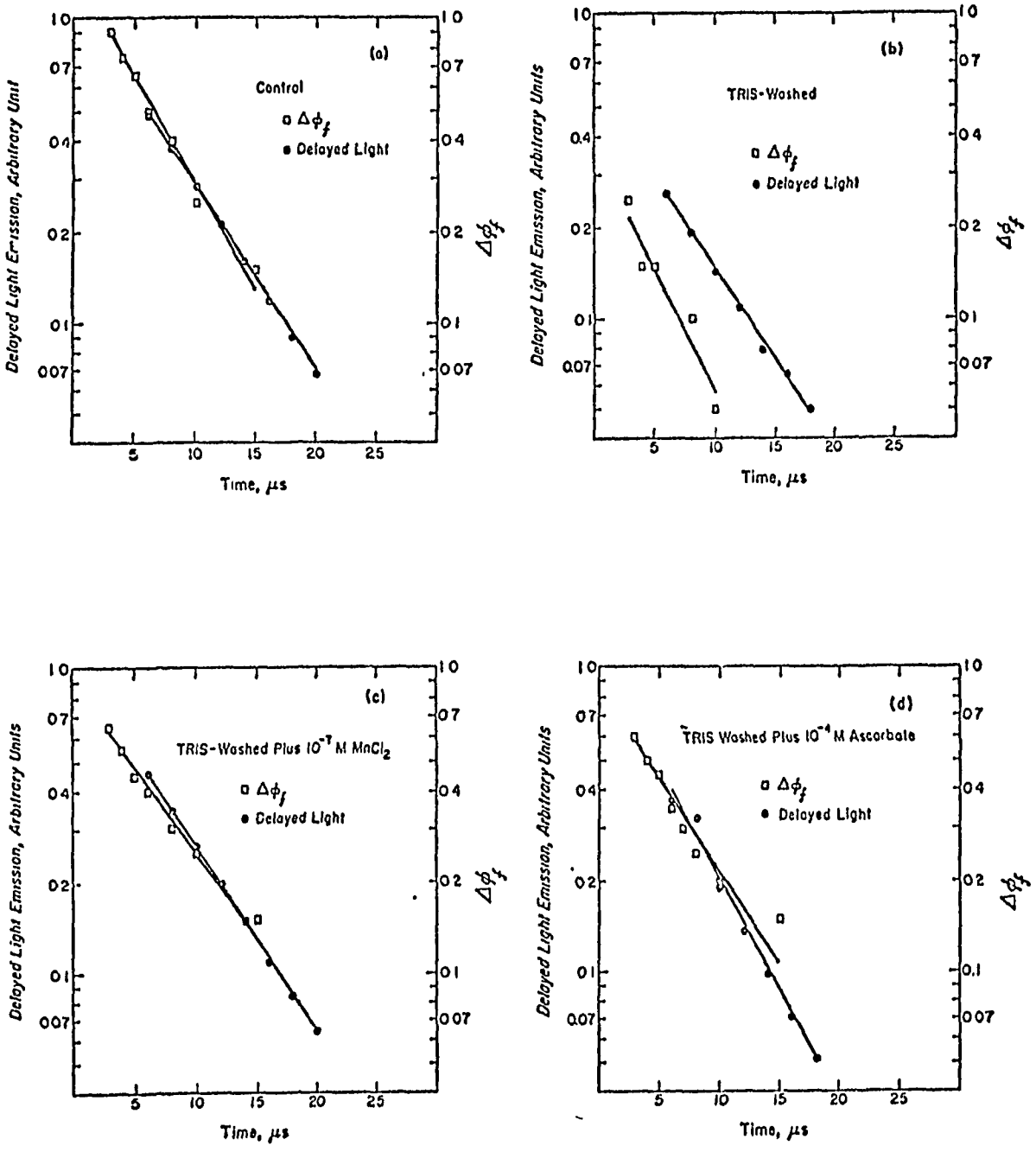
Earlier, we suggested that Mn^{2+} may donate electrons to P_{680} via Z (we shall call it Z_1). Due to differences with other donors [55], we suggest that other donors donate electrons to Z_2, which must be responsible for ESR signal II_{vf}. Thus, the Photosystem II electron flow reactions may be written as:



where Z_1 and Z_2 are charge carriers between the site of TRIS block and P_{680}. This interpretation for the TRIS effect is in agreement with those from absorption data [62, 63]. Contrary to this, Velthuys and Amesz [64],

Fig. 11. The most rapid delayed light emission decay component and variable chlorophyll a fluorescence yield in the 3 to 20 μs range.

Logarithmic plot of the most rapidly decaying component of delayed light emission beginning at 6 μs after the excitation flash and the variable fluorescence yield, $\Delta\phi_f = \phi_f(\max) - \phi_f(t)$, beginning at 3 μs. The delayed light emission decay component was obtained from decay curves by subtracting off the contribution from slower decay components and $\Delta\phi_f$ was obtained from fluorescence rise curves. All data are from pea chloroplasts at a chlorophyll concentration of 5 μg/ml receiving illumination at a rate of 1 flash/s. $\Delta\phi_f$ is given in terms of ϕ_0, the yield of fluorescence prior to an actinic flash, and delayed light emission is in arbitrary units, but normalized in (a) to have the same value as $\Delta\phi_f$ at 6 μs. Delayed light emission in (b), (c), and (d) is on the same scale as in (a). (a) control, (b) TRIS washed, (c) TRIS washed plus 10^{-7} M $MnCl_2$, and (d) TRIS washed plus 10^{-4} M ascorbate.


(a)
Control
□ $\Delta\phi_f$
• Delayed Light
Delayed Light Emission, Arbitrary Unit
$\Delta\phi_f$
Time, μs
(b)
TRIS-Washed
□ $\Delta\phi_f$
• Delayed Light
Delayed Light Emission, Arbitrary Units
$\Delta\phi_f$
Time, μs
(c)
TRIS-Washed Plus 10^{-7} M $MnCl_2$
□ $\Delta\phi_f$
• Delayed Light
Delayed Light Emission, Arbitrary Units
$\Delta\phi_f$
Time, μs
(d)
TRIS Washed Plus 10^{-4} M Ascorbate
□ $\Delta\phi_f$
• Delayed Light
Delayed Light Emission, Arbitrary Units
$\Delta\phi_f$
Time, μs

observing changes in fluorescence after rapid additions of dithionite, had concluded that TRIS washed chloroplasts can only store a single positive charge on the donor side of Photosystem II.

The 30 μs decay component of delayed light emission is sensitive to TRIS washing only on the second and subsequent flashes. In the above scheme, this would be after Z_2 has become oxidized. We suggest that the 30 μs components reflects the stabilization of charge when moving from Z_1 to Z_2, which becomes modified to a 60 μs component when Z_2 becomes oxidized. Another possibility is that after two or more flashes Z_1 and Z_2 become oxidized and reactions which stabilize the P^+_{680} charge are no longer kinetically observable in this time range in delayed light emission decay. Instead, a stabilization reaction involving the Q^- charge now becomes perceptible in this time range. The last hypothesis seems most reasonable for explaining the existence of a 60 μs component in chloroplasts treated with high concentrations of hydroxylamine in the light, which have their $Z_1P^+_{680} \longrightarrow Z^+_1P_{680}$ reaction completely blocked (see Chapter IV).

In a recent paper [65]* the delayed light emission in the 0.2 to 20 μs time range has been studied in intact cells of Chlorella following short saturating flashes. A decay component of approximately 1 μs lifetime was observed along with a 10-17 μs decay component. This 1 μs component was most prominent in anaerobic preparations and has been attributed to the $ZP^+_{680} \longrightarrow Z^+P_{680}$ reaction in Chlorella. In our chloroplast preparations we observed a 6 μs rise of the fluorescence yield, and we believe reflects the $ZP^+_{680} \longrightarrow Z^+P_{680}$ reaction. It is felt these differences in results lie in the differences between the intact cells and chloroplasts. For example, differences in the relaxation time of the oxygen evolving S-states, in

* This paper appeared during the preparation of this thesis.

intact cells and chloroplasts, are known to exist [11, see Chapter IV *for further discussion*].

In conclusion, data have been presented here which suggest that conditions which favor high P_{680}^{+} concentrations result in enhanced delayed light emission and virtually eliminate the variable yield of fluorescence. This result confirms the hypothesis that delayed light emission in the microsecond time range is due to a back reaction of P_{680}^{+} and Q^{-} and that P_{680}^{+} acts as a quencher of fluorescence.

CHAPTER IV

EFFECTS OF HYDROXYLAMINE AND SILICOMOLYBDATE ON DELAYED LIGHT EMISSION AND IN THE RISE IN CHLOROPHYLL A FLUORESCENCE YIELD

A. Introduction

The reaction center of Photosystem II consists of two fundamental components: P_{680}, the reaction center chlorophyll a and primary electron donor, and Q (or X-320), the "primary" electron acceptor. Upon sbsorption of a quantum of light the reaction center undergoes a charge separation with P_{680} becoming oxidized [4, 66] and Q reduced [12]. It was suggested that microsecond delayed light emission is generated by the charge recombination of P_{680}^{+} and Q^{-} [20, 24] . Loss of Photosystem II oxidation reduction energy by delayed light emission is prevented by stabilization of the charge by the following reaction [47]: $ZP_{680}^{+}Q^{-} \longrightarrow Z^{+}P_{680}Q^{-}$, where Z is a secondary electron donor on the oxygen evolving side of Photosystem II. It has also been suggested that the early phases of the microsecond delayed light emission decay should be related to steps in this charge stabilization [47, 48].

Hydroxylamine (NH_2OH) affects Photosystem II activity and, in particular it inhibits oxygen evolution [53]. If chloroplasts are treated with 2 mM NH_2OH in the dark, then oxygen evolving capacity is destroyed, but NH_2OH can still act as an electron donor to photosystem II. However, if chloroplasts are treated with 2 mM NH_2OH in the light, both oxygen evolving activity and the ability for NH_2OH to donate electrons to Photosystem II are eliminated [53]. On the basis of the microsecond rise of chlorophyll a fluorescence yield measured in intact cells of Chlorella treated with

NH_2OH in the dark [15, 67], Den Haan and coworkers proposed that NH_2OH eliminates the $ZP^+_{680} \longrightarrow Z^+P_{680}$ charge stabilization reaction and in place of Z another donor, D, reduces P^+_{680} with a halftime of 25 μs. Lavorel [47] measured the microsecond decay of delayed light in Chlorella and found a component with 5-10 μs lifetime to decrease upon NH_2OH addition, and components with lifetimes of 50-70 μs and 110-300 μs to increase. None of the previous publications provided data on both delayed light decay and chlorophyll a fluorescence rise to allow precise correlations between the two phenomena and, moreover, most of the work was restricted to intact algal cells.

In this chapter, we report both the rise in chlorophyll a fluorescence yield and the decay of delayed light emission in the microsecond time range in isolated Alaska pea chloroplasts. Since the fluorescence yield rise is related to the decreasing concentration of P^+_{680} [15, 56], we can monitor how various treatments affect this Photosystem II reaction. This information is essential for interpreting delayed light emission. NH_2OH treatment, in light or dark, of chloroplasts was used to alter the Photosystem II charge stabilization reaction on the oxygen side of Photosystem II. The sites of electron donation by $MnCl_2$ and diphenyl carbazide were investigated. Silicomolybdate has been shown to accept electrons between Q^- and the DCMU inhibition site [27, 28]. We added silicomolybdate to chloroplasts as a means of competing with P^+_{680} for the Q^- charge. The effects of this treatment on microsecond delayed light emission and chlorophyll a fluorescence yield rise was also investigated.

B. Materials and Methods

Chloroplasts from Alaska pea leaves were used in all of these experiments. The chloroplast isolation procedure is given in Chapter II. Except

for oxygen evolution measurements, chloroplasts were diluted immediately before use to a chlorophyll concentration of 5 μg/ml with media containing 0.4 M sucrose, 40 mM KCl and 50 mM sodium phosphate (pH = 7.8). All experiments were carried out at 25 °C.

Hydroxylamine treatments of chloroplasts were as follows:

(a) low concentration of NH_2OH (50 μM) was added to chloroplasts at a chlorophyll concentration of 1 mg/ml in complete darkness; it was not washed out; or,

(b) chloroplasts at a chlorophyll concentration of 300 μg/ml were incubated with 2 mM NH_2OH in white light (2 mW/cm^2) for 15 min at 25 °C; these treated chloroplasts were centrifuged and washed twice with buffer and resuspended to a chlorophyll concentration of 5 μg/ml [8]; or,

(c) high concentration of NH_2OH (2 mM) was added to chloroplasts; all conditions were as described in (b) except that the treatment was in complete darkness.

The effectiveness of the high NH_2OH concentration treatments was tested by measuring, under saturating continuous illumination, the rate of oxygen evolution with ferricyanide as an electron acceptor, using a Yellow Springs Instrument Clark electrode and Model 53 oxygen monitoring system.

The time course of chlorophyll a fluorescence (Fluorescence induction) under continuous illumination was measured with a spectrofluorometer [68] as described by Munday and Govindjee [69]. Oxygen evolution under flashing light conditions (O_2/flash as a function of number of flashes in a series of flashes) was measured as described in Chapter II.

The apparatus used for measuring delayed light emission and the rise in chlorophyll a fluorescence yield in the microsecond time range is also described in Chapter II.

The protocol of Zankel [59] was followed for the quantum yield of delayed light emission (ϕ_{DLE}) measurements. The chlorophyll *a* fluorescence rise measurement, needed for the ϕ_{DLE} calculation, was made by replacing the nitrogen laser with light from an incandescent lamp, passed through a two-inch water filter, a Corning CS4-96 glass filter and a camera shutter. The intensity of this continuous broad-band blue excitation was 1.5 mW/cm^2 incident at the sample. As required for determining ϕ_{DLE}, all experimental conditions, except the excitation source, were kept identical for both delayed light emission and chlorophyll *a* fluorescence measurements.

For experiments in which silicomolybdate was present, the following protocol was used: (1) chloroplasts were illuminated for 10 s with the nitrogen laser at a flash rate of 32 Hz; (2) during this illumination, either silicomolybdate or ferricyanide was added, followed by DCMU; and (3) after one minute dark adaptation, a single laser flash was given followed by measurements of the delayed light emission decay. This particular procedure for the use of silicomolybdate was followed since it was reported earlier [28] that silicomolybdate accepted electrons before the DCMU block most efficiently if it was added in the light prior to DCMU. For measuring electron flow in these samples, a Clark oxygen electrode (described in Chapter II) was used. The same protocol, as just given, was followed except the 10 s illumination was made with the continuous light used for oxygen evolution measurements.

Recrystallized NH_2OH was used in this work and the pH of its solution was adjusted to 7.8 at 25 °C. Fresh solutions of NH_2OH were prepared on the day of the experiment. Silicomolybdate was obtained from K & K Laboratories and dissolved in warm water.

C. Results

1. Chloroplasts Treated with Low Concentration of NH_2OH

Treatment of Chlorella and spinach chloroplasts in dark with micromolar concentrations of NH_2OH does not destroy oxygen evolution but alters its O_2 flash yield dependence [70]. We show here the effects of low concentration of NH_2OH [50 μM (1 NH_2OH/20 chlorophyll); see treatment (a) under Methods] on the microsecond chlorophyll a fluorescence yield rise and microsecond delayed light emission decay, and flash number dependence in Alaska pea chloroplasts.

The delayed light decay, shown in Fig. 12, is similar to that of the control. The rise in chlorophyll a fluorescence yield in the microsecond time range is also identical to the control rising exponentially with a lifetime of 6 μs to a value of ~3.0 ϕ_0 (data not shown). (ϕ_0 is the level of fluorescence yield prior to an excitation flash.)

Although the 50 μM NH_2OH treatment has no effect on the delayed light emission decay, it does affect the dependence of the intensity of delayed light emission at 60 μs (after the flash) on flash number (Fig. 13). In control chloroplasts, delayed light emission peaks on the third flash and follows highly damped oscillations of period four in agreement with earlier results [49, 59]. Chloroplasts with 50 μM NH_2OH present have maximal delayed light emission on the sixth flash, a three-flash delay compared to control. Similar effects of 50 μM NH_2OH have been reported earlier [71], however, only for delayed light emission at 50 ms after a flash.

For comparison purposes, the yield of oxygen per flash as a function of flash number is shown in Fig. 14. The control exhibits the normal oxygen evolution flash pattern while the 50 μM NH_2OH treated sample has its peak of

Fig. 12. Delayed light emission decay from 6 to 100 μs in chloroplasts with and without 50 μM NH_2OH.

Logarithmic plot of delayed light emission decay from 6 to 100 μs after a single 10 ns flash following 5 min dark adaption. Decays are shown for control Alaska pea chloroplasts (o—o—o) and those containing 50 μM NH_2OH (1 NH_2OH per 20 chlorophyll) (□—□—□). Measurements were made with samples containing 5 μg chlorophyll/ml.

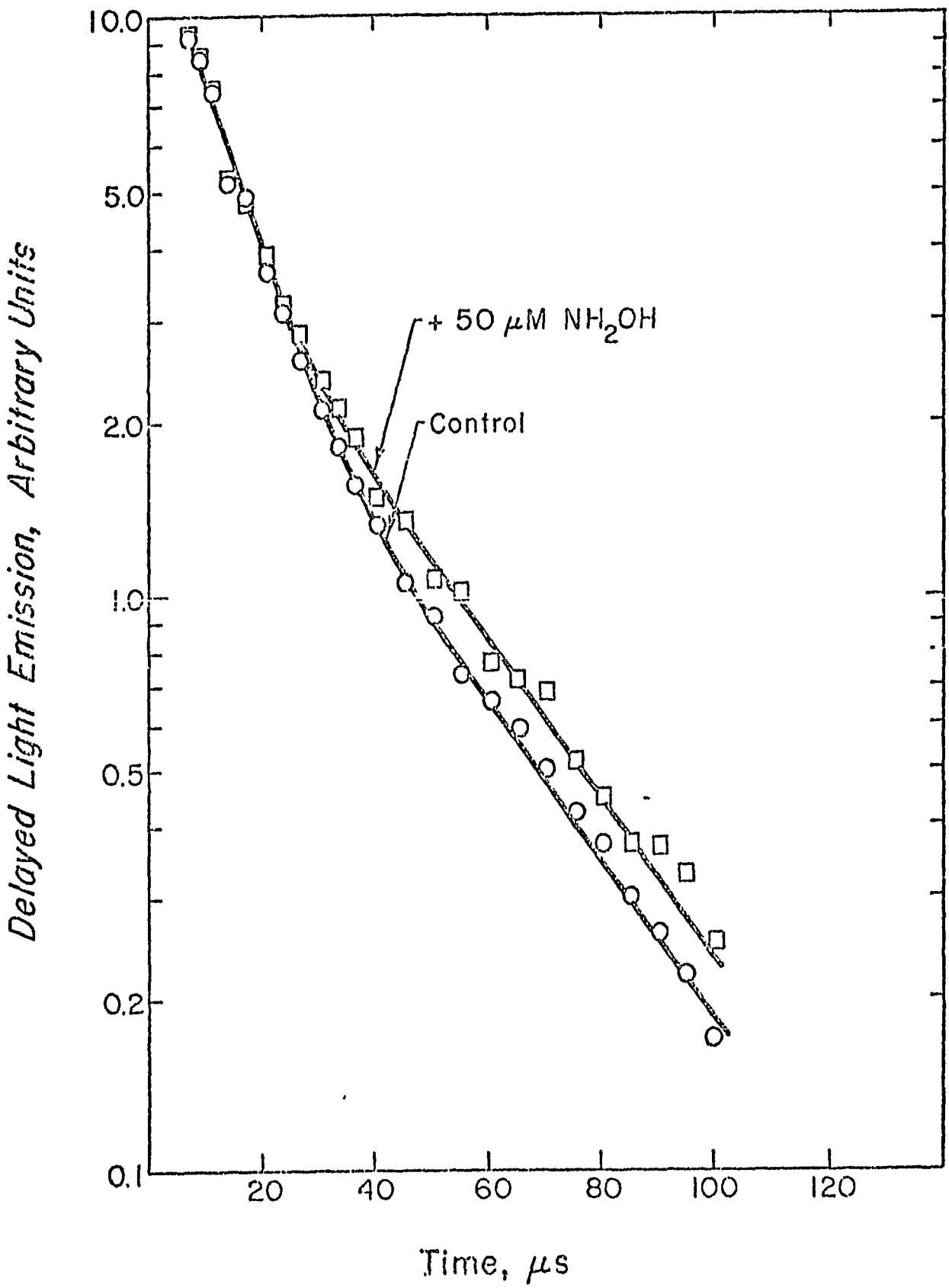

10.0
5.0
2.0
1.0
0.5
0.2
0.1
20
40
60
80
100
120
+ 50 μM NH2OH
Control
Delayed Light Emission, Arbitrary Units
Time, μs

Fig. 13. Flash number dependence of delayed light emission at 60 μs.

Delayed light emission intensity at 60 μs after the flash versus flash number following a 10 min dark adaptation. Flashes were given at a rate of one per second to untreated Alaska pea chloroplasts (o—o—o) and those containing 50 μM NH_2OH (1NH_2OH per 20 chlorophyll) (□—□—□). Measurements were made with samples containing 5 μg chlorophyll/ml.

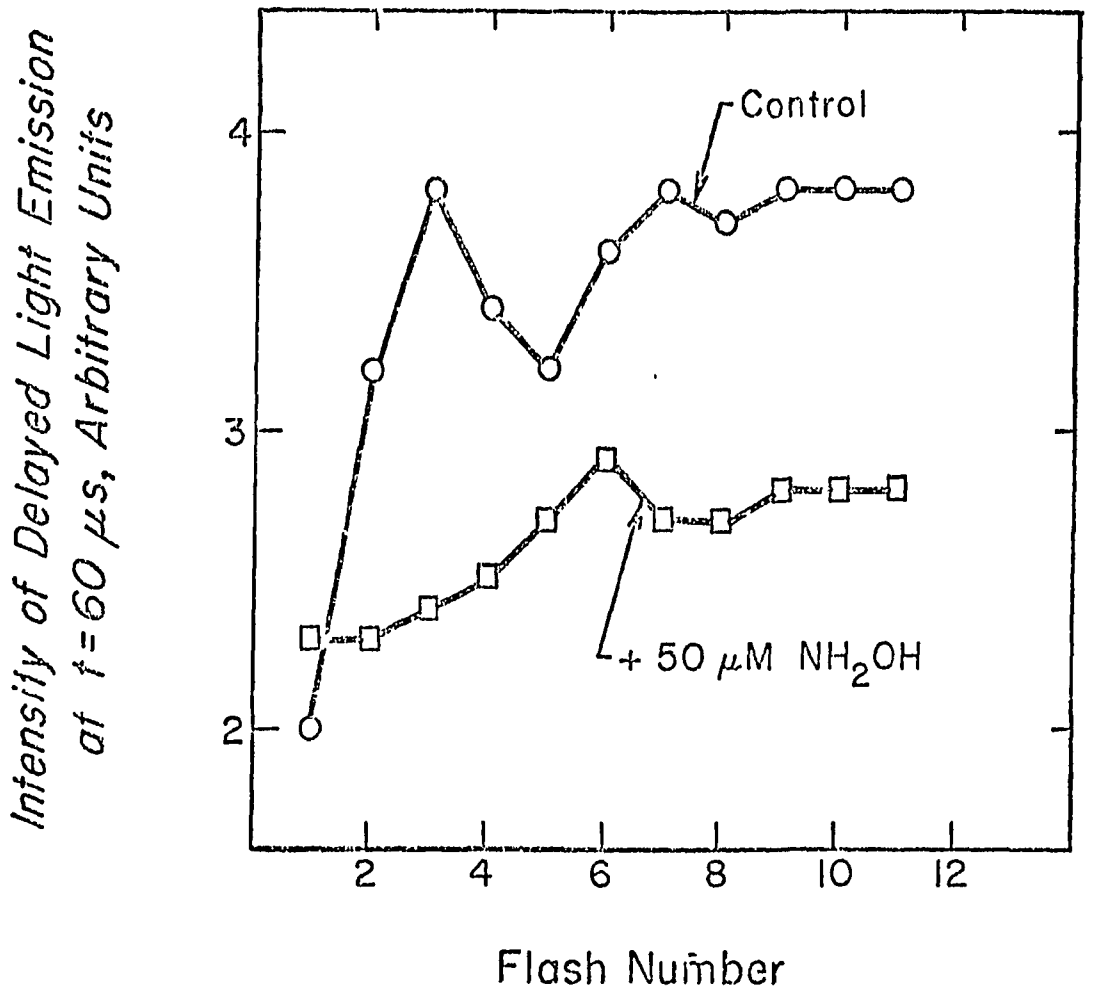
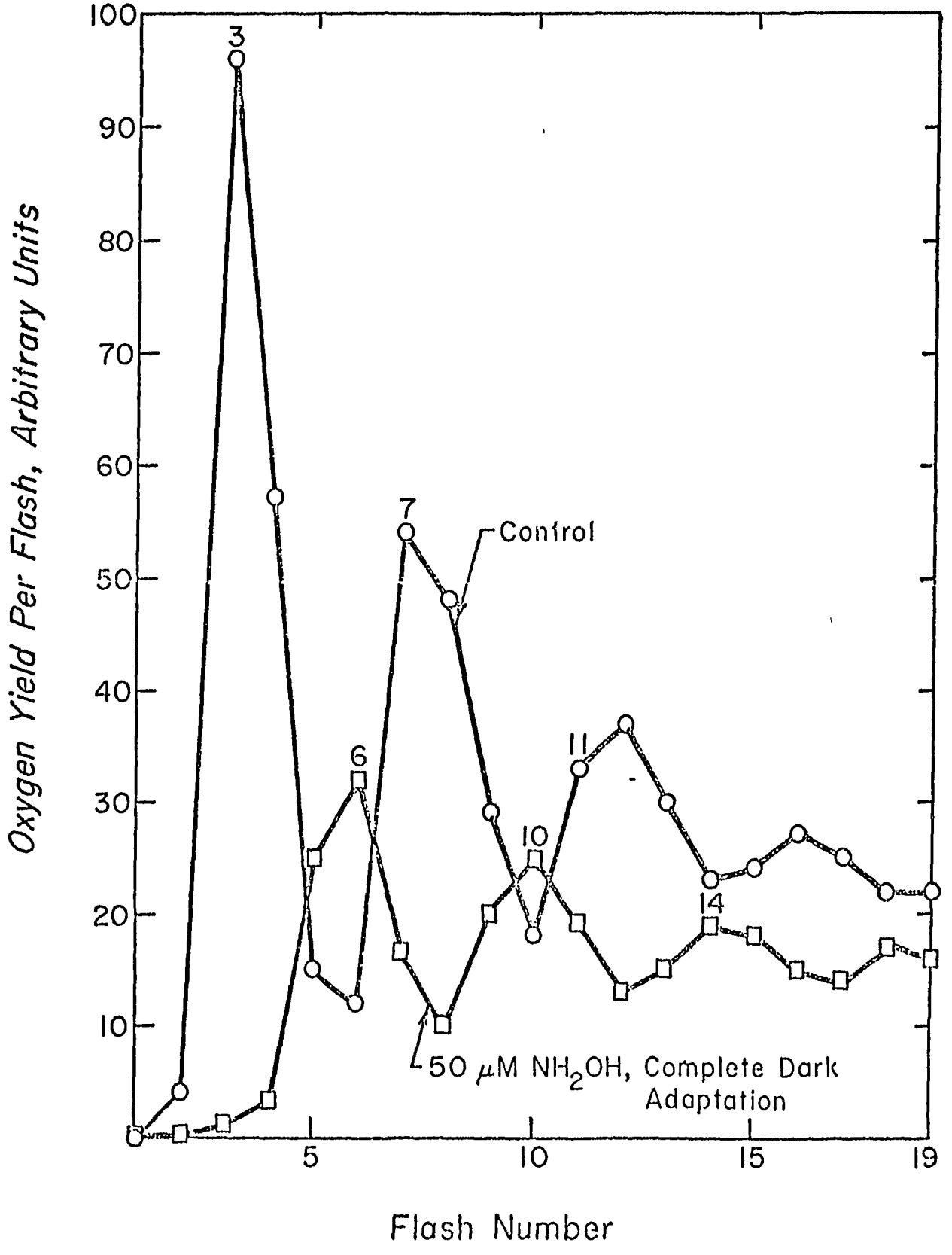

Intensity of Delayed Light Emission
at t = 60 μs, Arbitrary Units
Control
+ 50 μM NH2OH
4
3
2
2
4
6
8
10
12
Flash Number

Fig. 14. Oxygen yield flash number dependence of chloroplasts with and without 50 μM NH_2OH.

Oxygen yield per flash versus flash number in Alaska pea chloroplasts following a ten minute dark adaptation. Measurements were made at a chlorophyll concentration of 1 mg/ml with (□—□—□) or without (o—o—o) 50 μM NH_2OH present. Flashes were given at a rate of 1 flash per second.


Oxygen Yield Per Flash, Arbitrary Units
100
90
80
70
60
50
40
30
20
10
3
7
6
11
10
14
Control
50 μM NH2OH, Complete Dark Adaptation
5
10
15
19
Flash Number

oxygen evolution delayed until the sixth flash and, thereafter, it follows a period of four oscillation. These changes in the oxygen yield flash conform with an earlier account [70].

2. Chloroplasts Treated in the Light with High Concentrations of NH_2OH

Treatment of chloroplasts under illumination with 2 mM NH_2OH (6.6 NH_2OH per chlorophyll) destroys oxygen evolution and causes NH_2OH to become incapable of donating electrons to Photosystem II [53]. Previous measurements of delayed light emission [47] and chlorophyll a fluorescence yield rise [15] were made in two different laboratories on algal cells and, obviously, on separate samples treated with NH_2OH in the dark. However, only delayed light emission was reported by Haveman and Lavorel [48] in chloroplasts. We report both these measurements in the same sample of pea chloroplasts treated with 2 mM NH_2OH in the light (as described in (b) under Methods).

In order to test the effectiveness of the 2 mM NH_2OH treatment under illumination, the chlorophyll a fluorescence transient under continuous illumination was used to test electron flow in Photosystem II [26]; 1 mM NH_2OH (200 NH_2OH per chlorophyll molecule) was unable to restore the normal fluorescence transient in these samples (data not shown); thus NH_2OH could not efficiently donate electrons to Photosystem II. The decay of delayed light emission is shown as a semilogarithmic plot in Fig. 15 and in terms of exponential lifetimes and amplitudes in Table 4, line 2. The rapidly decaying component with $\tau \approx 6$ μs is greatly inhibited by NH_2OH treatment in the light while a second slower component is enhanced and decays with a lifetime of 50-60 μs versus 32 μs in the control. Also, the yield of delayed light emission, represented by the area under the decay curve, is

Fig. 15. Delayed light emission decay from 6 to 100 μs in chloroplasts treated with NH_2OH in the light.

Logarithmic plot of delayed light emission for 6 to 100 μs after a single excitation following 5 min dark adaptation. Decays are shown for control Alaska pea chloroplasts (o—o—o) and those treated with 2 mM NH_2OH per 300 μg chlorophyll/ml in the light for 15 min (□—□—□). The NH_2OH was then washed out and the delayed light measurements were made on samples having chlorophyll concentration of 5 μg/ml.

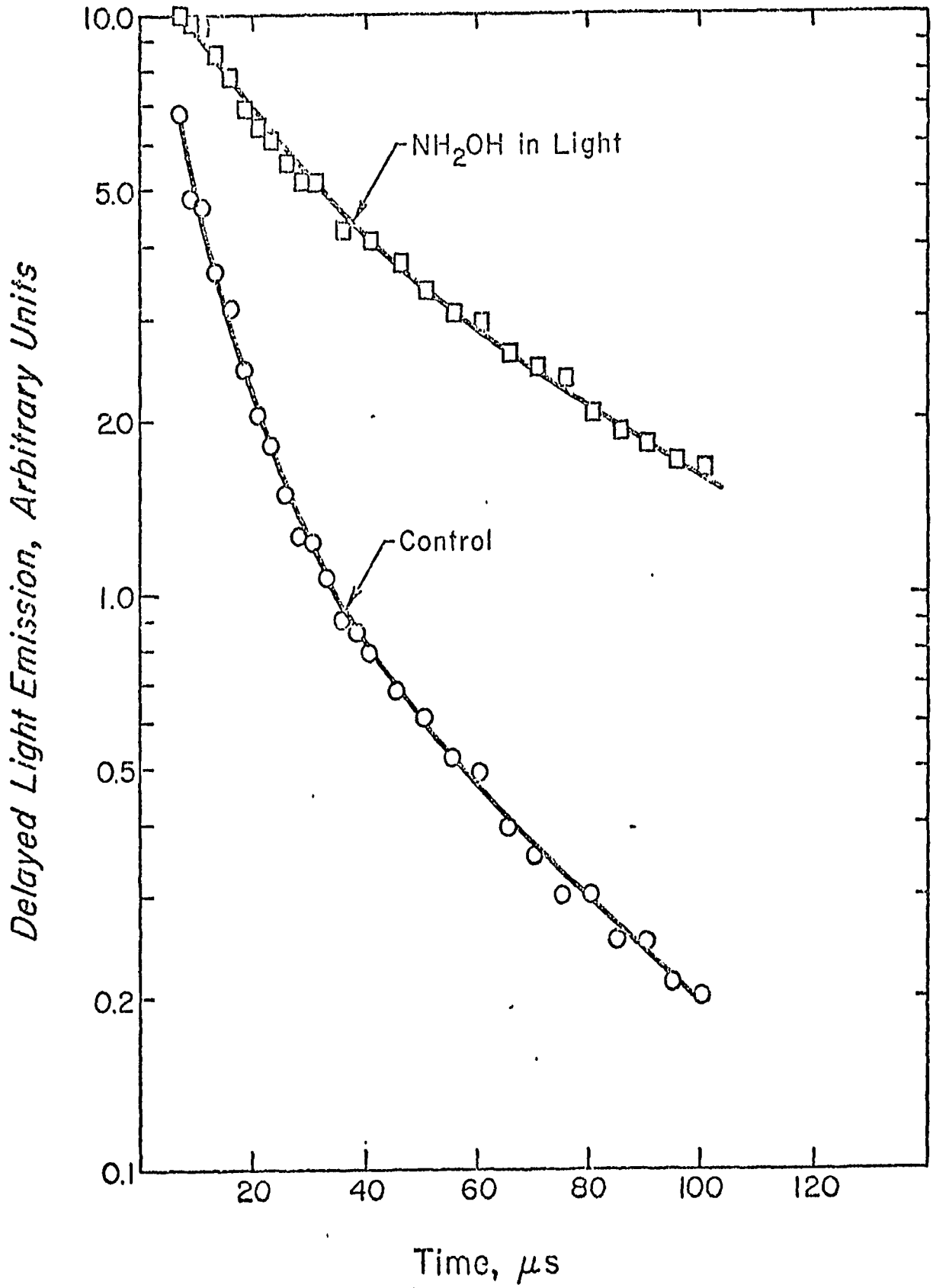

10.0
5.0
2.0
1.0
0.5
0.2
0.1
20
40
60
80
100
120
NH_2OH in Light
Control
Delayed Light Emission, Arbitrary Units
Time, μs

TABLE 4.

Decay Characteristics of Delayed Light Emission in the 6-100 μs Range for Hydroxylamine Treated Chloroplasts.

Standard graphical procedures were applied to semilog plots of delayed light emission decays to calculate amplitudes (α) and lifetimes (τ). Amplitude values are given in percentages of the extrapolated zero time value for delayed light emission, $\Sigma\alpha_i = 100\%$, and τ values are given in microseconds. Typical measurement errors are given as τ one standard deviation unit. The areas under the curve are normalized to one for the control sample. Alaska pea chloroplasts at a chlorophyll concentration of 5 μg/ml were used and specific treatment procedures are given in Materials and Methods.

Line	Sample Conditions	α_1	τ_1, μs	α_2	τ_2, μs	Area
1	Control	81	6	19	32	1
2	Treated with 2 mM NH_2OH in light	35 ± 5	7 ± 1	65 ± 5	52 ± 6	2.6
3	Treated with 2 mM NH_2OH in light plus 10 μM $MnCl_2$	29	6	71	62	2.7
4	Treated with 2 mM NH_2OH in light plus 1 mM ascorbate and 10 μM benzidine	32	6	68	53	2.5
5	Treated with 2 mM NH_2OH in light plus 1 mM ascorbate and 10 μM phenylenediamine	25	6	75	56	2.6
6	Treated with 2 mM NH_2OH in light plus 1 mM diphenyl carbazide	32	7	68	57	2.4
7	Treated with 2 mM NH_2OH in dark	26	7	74	46	2.5
8	Treated with 2 mM NH_2OH in dark plus 1 mM NH_2OH	37	7	63	45	2.0

increased by 2.6 fold. The rise in chlorophyll a fluorescence yield, after a single excitation flash, is shown in Fig. 16; hydroxylamine treatment in light eliminates the variable yield. The inhibition of the rise in fluorescence yield, having a 6 μs lifetime, indeed, corresponds with the decrease in the 6 μs of delayed light emission.

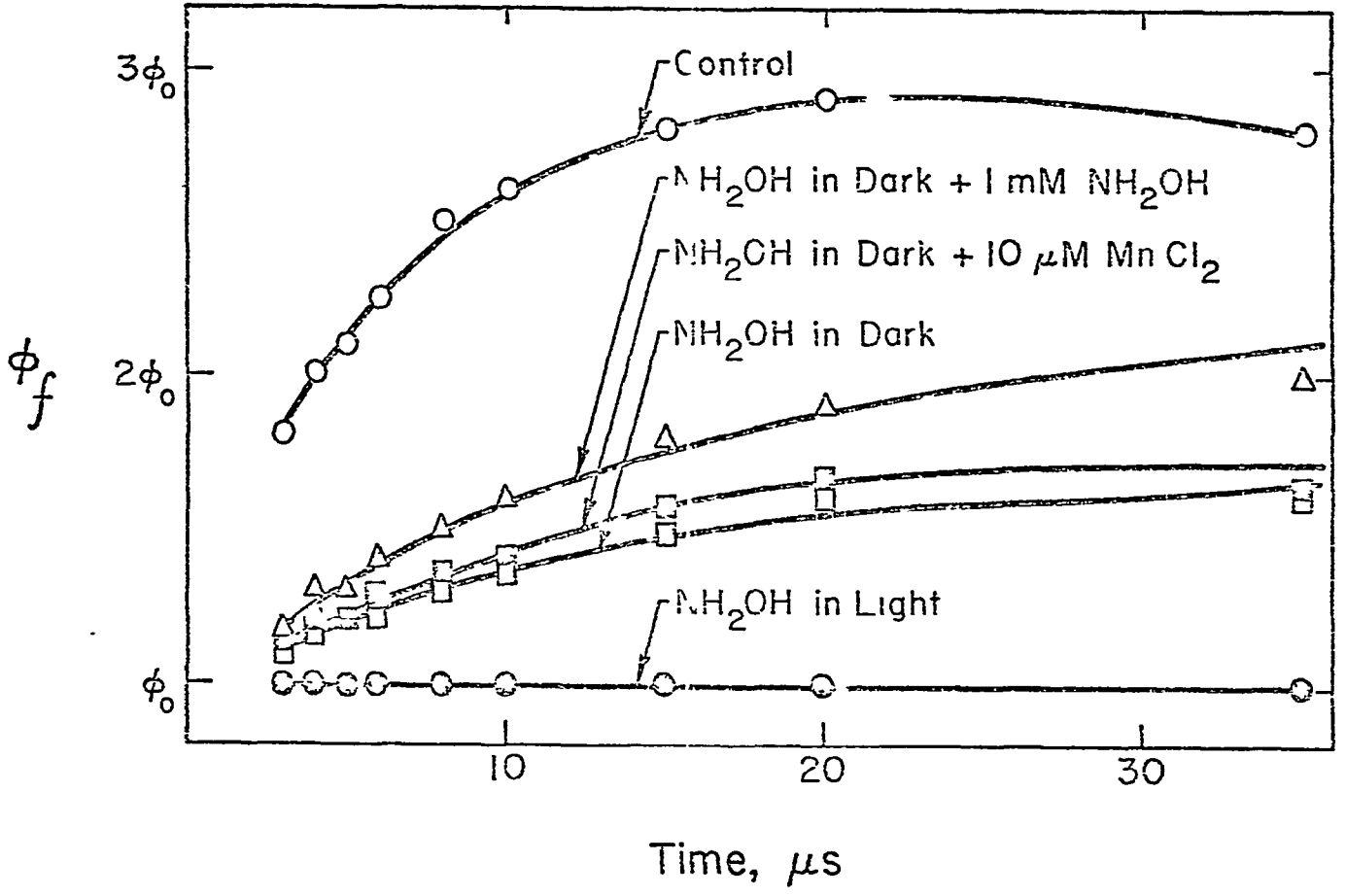
Since the high amount of delayed light emission and low chlorophyll a fluorescence yield are indicative of blocked electron flow from Z to P_{680}^{+} (see Discussion for detailed arguments), the effects of various Photosystem II electron donors ($MnCl_2$, reduced benzidine and phenylenediamine and diphenyl carbazide) on delayed light emission and chlorophyll a fluorescence yield rise were studied to check if any of these donors donated electrons direction to P_{680}^{+}. All of these electrons were unable to reverse the effects of NH_2OH (in light) on delayed light emission (see lines 3-6 of Table 4) and on the rise in fluorescence yield. The absence of a very slow rise in fluorescence yield in the presence of electron donors was verified by also doing the measurement in samples with 0.1 μM DCMU (1DCMU per 50 chlorophyll) present. (DCMU allows any slow rise in the fluorescence yield to be observed more readily since a decrease in fluorescence yield by Q^- decay is blocked.)

3. Chloroplasts Treated in the Dark with High Concentrations of NH_2OH

The treatment of chloroplasts in darkness (see (c) under Methods) with 1 mM NH_2OH (6.6 NH_2OH per chlorophyll) inhibits their capacity to evolve oxygen, but Photosystem II activity with NH_2OH as an electron donor is still possible [53]. As expected, the fluorescence transient under continuous illumination, used to test for electron flow in Photosystem II [26], is partially restored in these samples when 1 mM NH_2OH is added (data not shown).

Fig. 16. Chlorophyll a fluorescence yield rise in chloroplasts treated with NH_2OH.

Rise in chlorophyll a fluorescence yield (ϕ_f) after a single flash in Alaska pea chloroplasts diluted to a chlorophyll concentration of 5 µg/ml after pretreatment NH_2OH had been washed out. ϕ_0 is the level of fluorescence yield prior to an actinic flash. Data from chloroplasts treated with 2 mM NH_2OH per 300 µg chlorophyll/ml for 15 min in the dark or in the light are shown with various donors as indicated: control (o—o—o); treated with 2 mM NH_2OH in the light (●—●—●); treated with 2 mM NH_2OH in the dark (□—□—□), plus 10 µM $MnCl_2$ (■—■—■) and plus 1 mM NH_2OH (△—△—△).


3ϕ_o
2ϕ_o
ϕ_o
ϕ_f
Control
NH_2OH in Dark + 1 mM NH_2OH
NH_2OH in Dark + 10 μM $MnCl_2$
NH_2OH in Dark
NH_2OH in Light
10
20
30
Time, μs

Treatment with NH_2OH in the dark produces changes in delayed light decay similar to NH_2OH treatment under illumination. The 6 μs component is greatly inhibited while a 50-60 μs decay component is enhanced as is the yield of delayed light emission. The presence of 1 mM NH_2OH as an electron donor causes a slightly more rapid decay of delayed light emission (see Fig. 17 and lines 7 and 8 of Table 4).

The rise in chlorophyll a fluorescence yield is not completely eliminated by NH_2OH treatment in the dark. Even with NH_2OH washed out, a slow exponential rise (τ = 38 μs) is still observed, and with 1 mM NH_2OH present this rise in fluorescence yield becomes more rapid (τ= 18 μs); see Fig. 16. In order to facilitate the analysis of fluorescence yield data, the maximum value of the yield was established by the addition of 0.1 μM DCMU (not shown in figure). The residual slow rise in fluorescence yield is believed to be a consequence of a partial leak of electrons to P^+_{680} in samples treated with high concentrations of NH_2OH in the dark. $MnCl_2$ does not appear to be as good an electron donor as NH_2OH across this partial block (Fig. 16).

4. Elimination of the NH_2OH Enhancement of Delayed Light Emission

Treatment of chloroplasts in the light or dark with 2 mM NH_2OH results in a significant enchancement of delayed light emission (Figs. 15 and 17). If 0.1μM DCMU (1 DCMU per 50 chlorphyll) is added to samples treated with 2 mM NH_2OH in the dark or the light and if these samples are preilluminated with saturating continuous white light for two minutes or with several flashes of laser light (see below), then the enhancement of delayed light emission is eliminated; furthermore, the delayed light emission intensity drops below to control (Fig. 18). This preillumination effect on the NH_2OH treated samples occurs only if DCMU is present and is

Fig. 17. Delayed light emission decay from 6 to 100 μs in chloroplasts treated with NH_2OH.

Logarithmic plot of delayed light emission for 6 to 100 μs after a single flash following 5 min dark adaptation. Decays are shown from chloroplasts treated with 2 mM NH_2OH per 300 μg chlorophyll/ml for 15 min in the dark. The NH_2OH was washed out and the delayed light measurements were made on samples having a chlorophyll concentration of 5 μg/ml with (△—△—△) or without (□—□—□) 1 mM NH_2OH added as an electron donor and control (o—o—o).

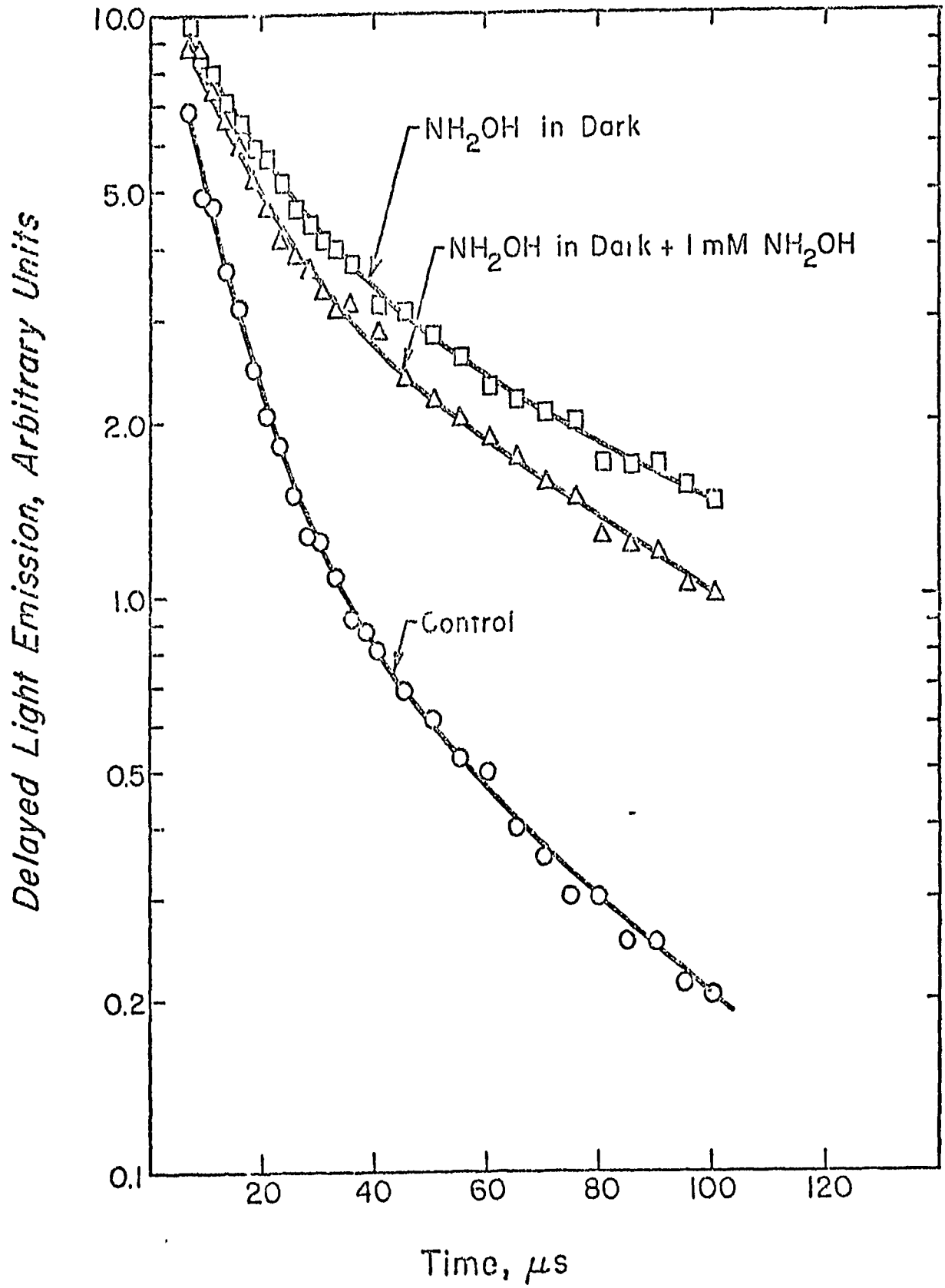
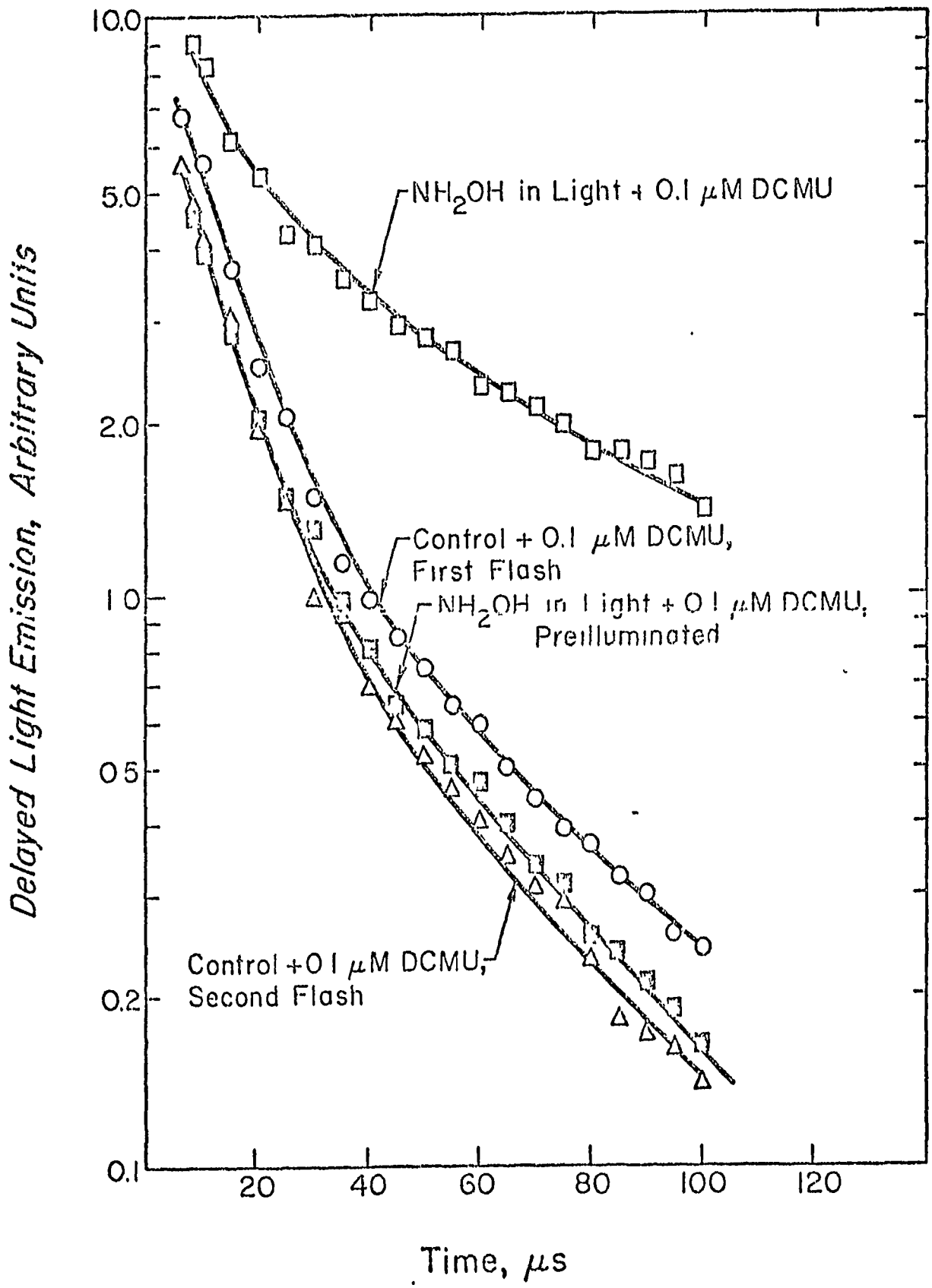

10.0
5.0
2.0
1.0
0.5
0.2
0.1
20
40
60
80
100
120
NH2OH in Dark
NH2OH in Dark + 1 mM NH2OH
Control
Delayed Light Emission, Arbitrary Units
Time, μs

Fig. 18. Delayed light emission decay from 6 to 100 μs in chloroplasts with DCMU and NH_2OH.

Logarithmic plot of delayed light emission for 6 to 100 μs after one or two flashes in Alaska pea chloroplasts treated with 2 mM NH_2OH per 300 μg chlorophyll/ml in the light for 15 min. The NH_2OH was then washed out and the delayed light measurements were made on samples having a chlorophyll concentration of 5 μg/ml. The various sample conditions are as follows: control plus 0.1 μM DCMU and decay after a single flash (o—o—o) and after a second flash given 66 ms after the first (△—△—△); treated with NH_2OH in the light plus 0.1 μM DCMU and decay after a single flash (□—□—□); treated with 2 mM NH_2OH in the light, plus 0.1 μM DCMU, preilluminated for two minutes in saturating continuous light and the decay after a single flash (■—■—■).

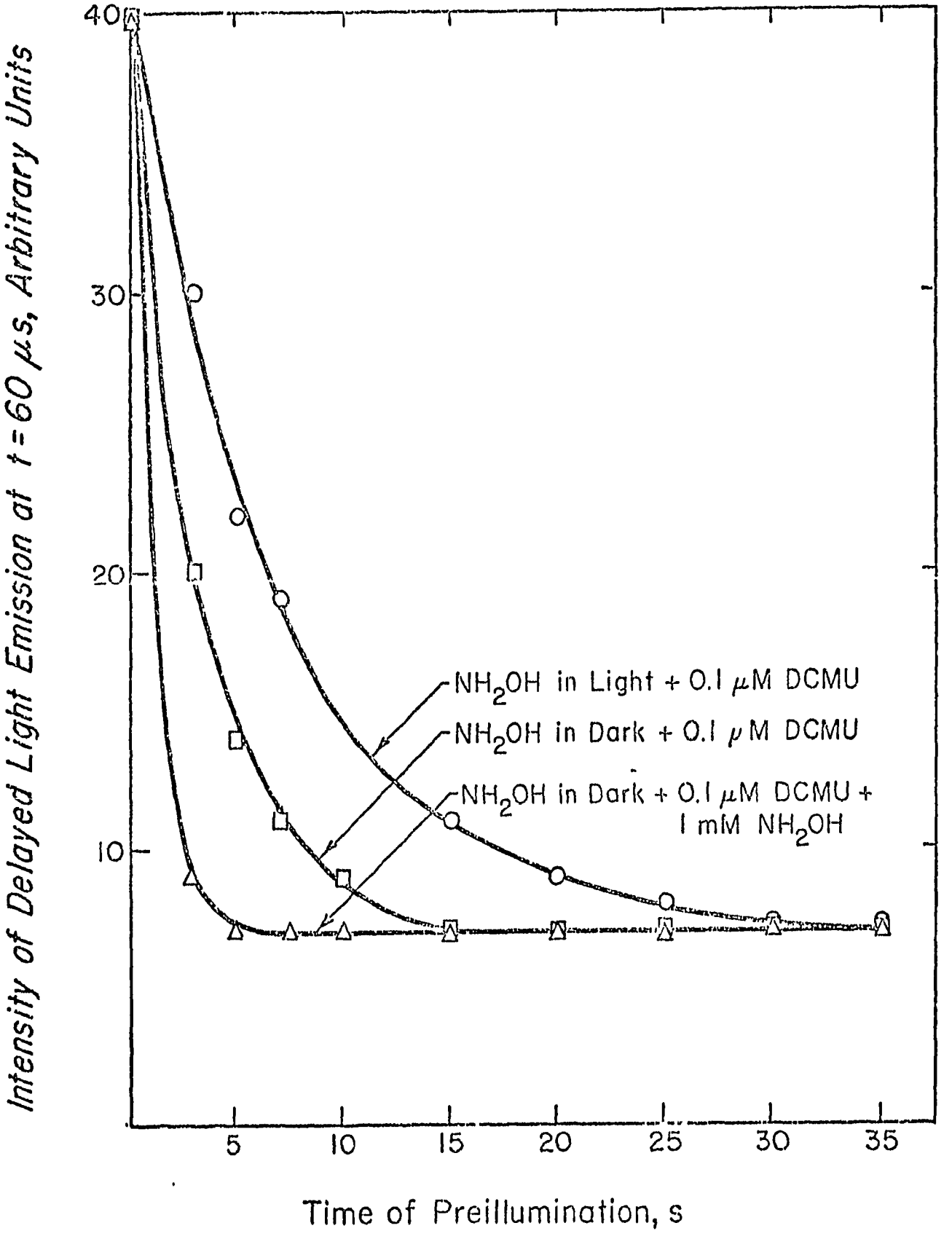

10.0
5.0
2.0
1.0
0.5
0.2
0.1
20
40
60
80
100
120
Delayed Light Emission, Arbitrary Units
Time, μs
NH2OH in Light + 0.1 μM DCMU
Control + 0.1 μM DCMU, First Flash
NH2OH in Light + 0.1 μM DCMU, Preilluminated
Control + 0.1 μM DCMU, Second Flash

believed to be due to preillumination causing reaction centers to change into a photoinactive state, most probably $P_{680}Q^-$. The same explanation was previously given for the loss of delayed light emission in the millisecond [26, 32] and second [25] time ranges in samples having both NH_2OH and DCMU present. To convert reaction centers into the photoinactive form ($P_{680}Q^-$) requires charge leakage across the NH_2OH block to P^+_{680} formed in the flash and can be characterized by observing the rate of onset of the preillumination effect. To accomplish this, the intensity of delayed light emission at 60 μs after an excitation pulse was measured following preillumination of the sample for various lengths of time with bursts of excitation laser pulses given at a frequency of 32 Hz (Fig. 19). As expected, samples having an incomplete blockage of electron flow from Z to P_{680} (treated with 2 mM NH_2OH in the dark) show an onset of the preillumination effect, with a $\tau_{\frac{1}{2}}$ of 2.2 s, which is unaffected by the addition of 10 M $MnCl_2$, but is accelerated ($\tau_{\frac{1}{2}}$ = 0.8 s) by the addition of 1 mM NH_2OH. The rate of onset of the preillumination effect in chloroplasts treated with 2 mM NH_2OH in the light (also shown in Fig. 19) is slower ($\tau_{\frac{1}{2}}$ = 5 s) and unaltered by the addition of 10 μM $MnCl_2$ or 1 mM NH_2OH (data not shown). The elimination of the enhancement of delayed light emission by preillumination is not reversed even in samples kept in darkness for as long as two hours at room temperature.

A residual amount of delayed light emission, however, remains even after many minutes of preillumination (Figs. 18 and 19). This residual delayed light emission signal is not artifactual since it can be completely eliminated by heating chloroplasts for ten minutes at 100 °C. The ratios of emission at 686 nm (mostly from Photosystem II) to that at 715 nm (both

Fig. 19. Preillumination effect on delayed light emission at 60 μs from NH_2OH treated chloroplasts.

The intensity of delayed light emission at 60 μs after a single flash in Alaska pea chloroplasts following preillumination with the excitation laser operating at 32 Hz. The samples are treated with 2 mM NH_2OH per 300 μg chlorophyll/ml in the light for 15 min plus 0.1 μM DCMU (o—o—o); treated with 2 mM NH_2OH per 300 μg/ml in the dark for 15 min plus 0.1 μM DCMU (□—□—□), and, plus 0.1 μM DCMU and 1 mM NH_2OH (△—△—△).


Intensity of Delayed Light Emission at t = 60 μs, Arbitrary Units
40
30
20
10
5
10
15
20
25
30
35
Time of Preillumination, s
NH2OH in Light + 0.1 μM DCMU
NH2OH in Dark + 0.1 μM DCMU
NH2OH in Dark + 0.1 μM DCMU + 1 mM NH2OH

Photosystems I and II) are the same for both the residual delayed light emission (see above) and for chloroplasts treated with 2 mM NH_2OH. Thus, the source of emission seems to be from the same photosystem, which is believed to be system II.

The chlorophyll a fluorescence yield in the microsecond range in samples that had been treated with 2 mM NH_2OH in the light and preilluminated with DCMU present was an invariant level of 2.1 ϕ_0 (data not shown).

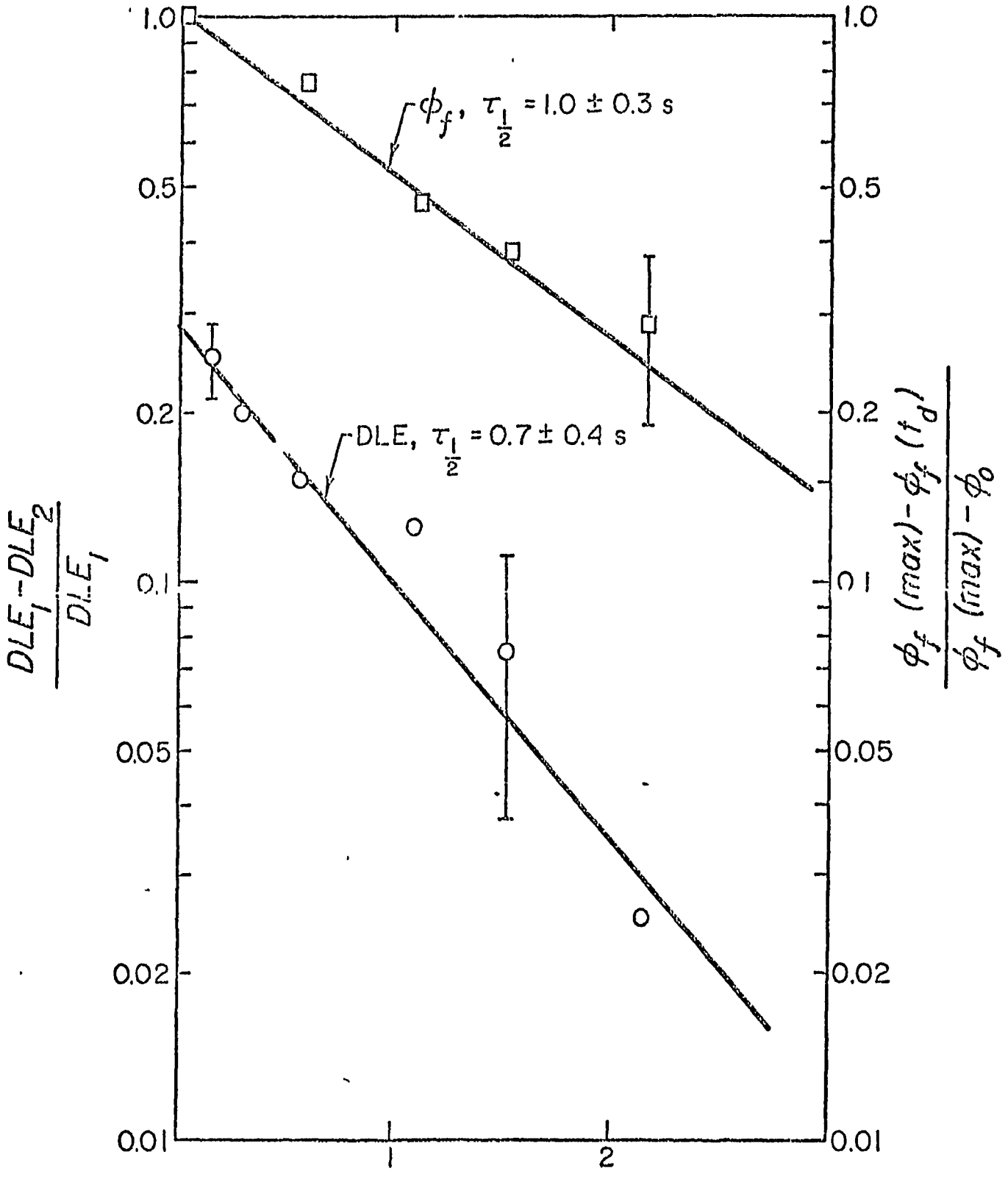
In control chloroplasts, reaction centers may be closed by DCMU addition followed by two excitation flashes, with a delay between these flashes shorter than the $Q^- \longrightarrow Q$ decay time. If the Q^- oxidation rate is monitored by following the decay in chlorophyll a fluorescence yield, after a single excitation flash, the halftime of Q^- oxidation with 0.1 μM DCMU present is 1.0 ± 0.3 s (upper curve, Fig. 20). Therefore, if two flashes are given with a time separation of much less than 1 s, the reaction centers will be in the closed form, $P_{680}Q^-$ at the time the second flash is given. As the delay time between the first and second flashes is decreased from 2.14 s to 133 ms, the delayed light emission intensity after the second flash decreased to about 70% of that occurring after the first flash (lower curve, Fig. 20). The level of delayed light emission after the second flash returns to the first flash level with a recovery halftime of 0.7 ± 0.4 s (lower curve, Fig. 20), which is about the same or slightly faster than the Q^- oxidation as indicated by the fluorescence yield decay.

5. Inhibition of Microsecond Delayed Light Emission by Silicomolybdate

If delayed light emission in the microsecond time range is generated from P^+_{680} and Q^- charge recombination, then removal of charge from Q^- should

Fig. 20. Double flash effect on chlorophyll a fluorescence yield and delayed light emission at 60 μs.

The delayed light emission intensity at 60 μs after the first flash (DLE_1) and the second flash (DLE_2) are plotted as $\frac{DLE_1 - DLE_2}{DLE_1}$ versus the time between the first and second flash. The chlorophyll a maximum fluorescence yield, $\phi_f(max)$, constant fluorescence yield, ϕ_0, and fluorescence yield, $\phi_f(t_d)$ at time t_d, after the first excitation flash are plotted as $\frac{\phi_f(max) - \phi_f(t_d)}{\phi_f(max) - \phi_0}$ versus the delay time between the first excitation flash and a second weak analytic flash. The error bars indicate the maximum possible errors. Samples were Alaska pea chloroplasts at a chlorophyll concentration of 5 μg/ml.


1.0
0.5
0.2
0.1
0.05
0.02
0.01
ϕ_f, $\tau_{\frac{1}{2}}$ = 1.0 ± 0.3 s
DLE, $\tau_{\frac{1}{2}}$ = 0.7 ± 0.4 s
$\frac{DLE_1 - DLE_2}{DLE_1}$
$\frac{\phi_f(max) - \phi_f(t_d)}{\phi_f(max) - \phi_0}$
1
2
Time Between 1st and 2nd Flashes, s

inhibit delayed light emission. Silicomoloybdate has been shown to accept [27, 28] electrons between Q^- and the DCMU inhibition site. Fig. 21 shows that with DCMU and ferricyanide present delayed light emission is high; however, with DCMU and 50 μM silicomolybdate (10 silicomolybdate per chlorophyll) added the delayed light emission is greatly reduced. In samples having DCMU and silicomolybdate present, initial electron flow rate through Photosystem II, determined by oxygen evolution rate within 5 s of illumination, was 60% larger than the rate observed in chloroplasts having only ferricyanide present. With 0.5 μM DCMU and 3 μM silicomolybdate (3 silicomolybdate per 5 chlorophyll) present the delayed light emission is inhibited and this inhibition can be reversed by illuminating the sample for one minute in saturating light (Fig. 21). This corresponds with the short-lived ability of silicomolybdate to accept electrons from Q^- in continuous light [28]. The inhibitory effect of 50 μM silicomolybdate on delayed light emission is large, but it cannot be reversed by illumination of the sample with continous light.

The chlorophyll a fluorescence yield after a single excitation flash rises normally in samples with 0.5 μM DCMU + 0.5 mM ferricyanide to 3 $\emptyset_0$, but remains at $\emptyset_0$ (the level of fluorescence in a control sample prior to excitation) with 3 μM silicomolybdate and at 0.5 $\emptyset_0$ with 50 μM silicomolybdate (Fig. 22). The loss of variable fluorescence yield with 3 μM silicomolybdate is reversed to a large extent by one minute illumination of the sample.

6. Quantum Yield of Delayed Light Emission

As described in Materials and Methods, a procedure similar to that used by Zankel [59] was followed to estimate the number of quanta emitted

Fig. 21. Delayed light emission decay from 6 to 100 μs in chloroplasts with silicomolybdate.

Logarithmic plot of delayed light emission in the 6 to 100 μs time range after a single excitation flash following 1 min dark adaptation. Chloroplasts were at a chlorophyll concentration of 5 μg/ml in the following media: 100 mM KCl, 5 mM $MgCl_2$ and 20 mM Tricine at pH 8.1. Decays are shown for chloroplasts treated as in Methods with: (o—o—o), 0.5 μM DCMU and 0.5 mM ferricyanide (FeCN); (△—△—△), 0.5 μM DCMU + 3 μM silicomolybdate (SiMo); (□—□—□) 0.5 μM DCMU + 50 μM SiMo; and (▲—▲—▲), 0.5 μM DCMU + 3 μM SiMo and illuminated for 1 min with saturating light.

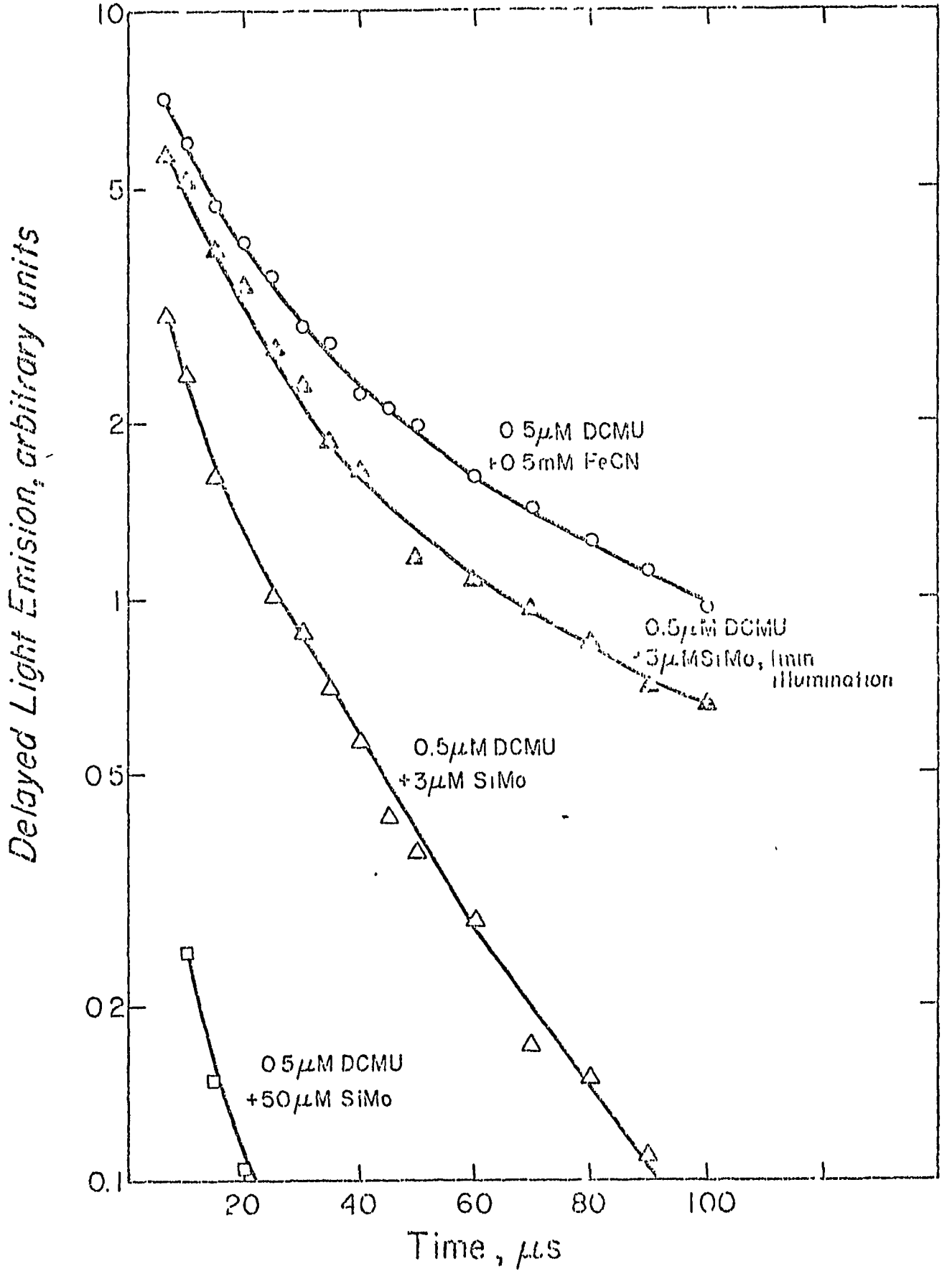
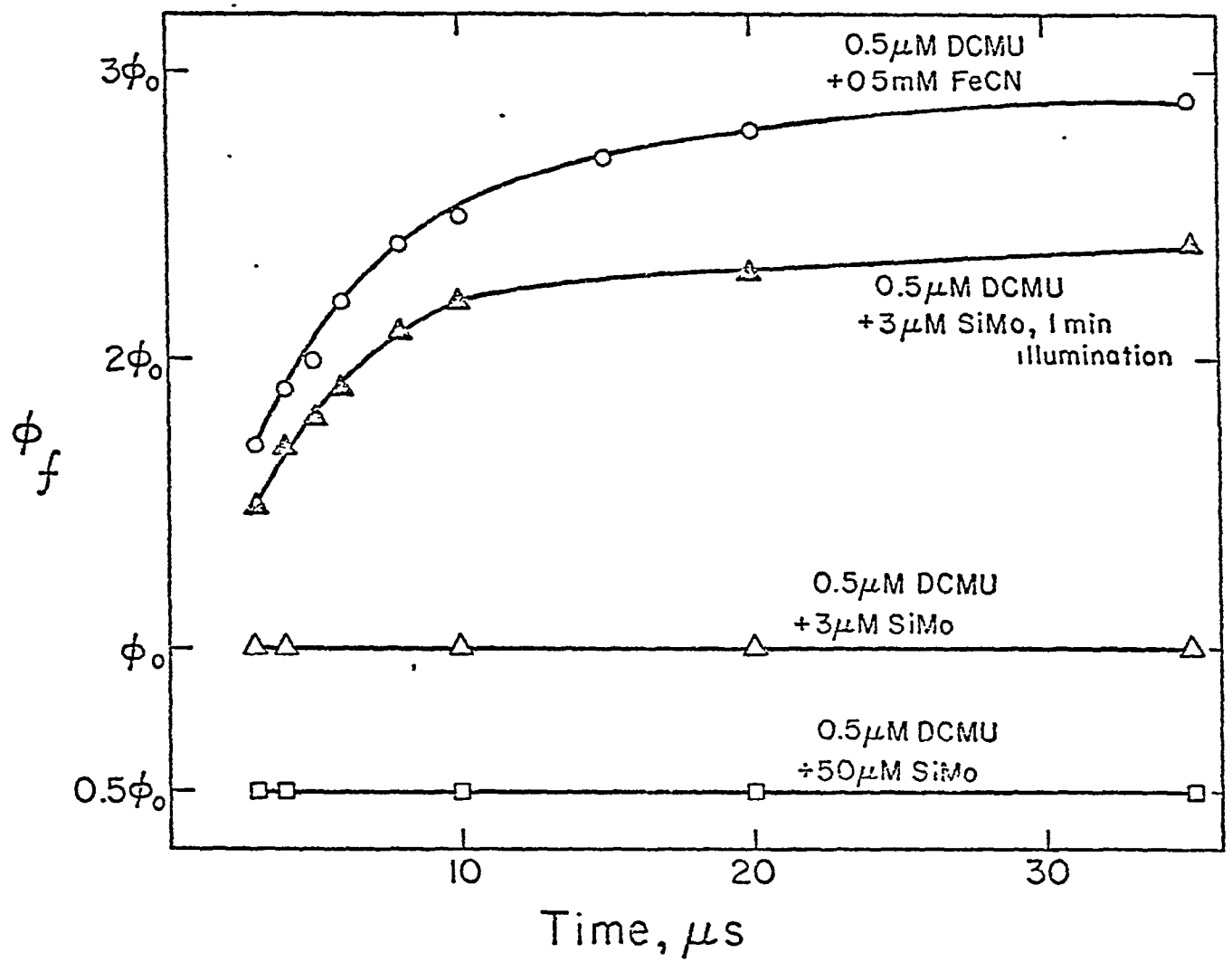

Delayed Light Emision, arbitrary units
10
5
2
1
0.5
0.2
0.1
20
40
60
80
100
Time, μs
0.5μM DCMU
+0.5mM FeCN
0.5μM DCMU
+3μM SiMo, 1min
illumination
0.5μM DCMU
+3μM SiMo
0.5μM DCMU
+50μM SiMo

Fig. 22. Chlorophyll a fluorescence yield rise in chloroplasts with silicomolybdate.

Rise in chlorophyll a fluorescence yield (ϕ_f) following a single excitation flash following 1 min dark adaptation. ϕ_0 is the level of fluorescence in an untreated sample prior to an actinic flash. Chloroplasts were at a chlorophyll concentration of 5 µg/ml in the following media: 100 mM KCl, 5 mM $MgCl_2$ and 20 mM Tricine at pH 8.1. Decays are shown for chloroplasts treated as in Methods with: (o—o—o), 0.5 µM DCMU and 0.5 mM ferricyanide (FeCN); (△—△—△), 0.5 µM DCMU + 3 µM silicomolybdate (SiMo); (□—□—□), 0.5 µM DCMU + 50 µM SiMo; and (▲—▲—▲), 0.5 µM DCMU + 3 µM SiMo and illuminated for 1 min with saturating light.


0.5μM DCMU
+0.5mM FeCN
0.5μM DCMU
+3μM SiMo, 1 min
illumination
0.5μM DCMU
+3μM SiMo
0.5μM DCMU
+50μM SiMo
3φ0
2φ0
φ0
0.5φ0
φf
10
20
30
Time, μs

as delayed light emission per quantum reaching a Photosystem II reaction center. These yields were determined for all delayed light decay components observed in the 6-400 μs time range. These are not total yields because components at times less than 6 μs have not been included. The error caused by truncating the area under the curve after 400 μs will only be a few percent due to the very low level of delayed light emission in the millisecond and second time range. For control chloroplasts, the delayed light emission quantum yield (ϕ_{DLE}) is 3.1×10^{-4} and for 2 mM NH_2OH treated samples with DCMU present, but not preilluminated, it is 1.1×10^{-3}. Both of these values for ϕ_{DLE} are significantly lower than the chlorophyll a fluorescence yield (ϕ_f) of 2.5×10^{-2} reported for low light intensity illumination [72, 73].

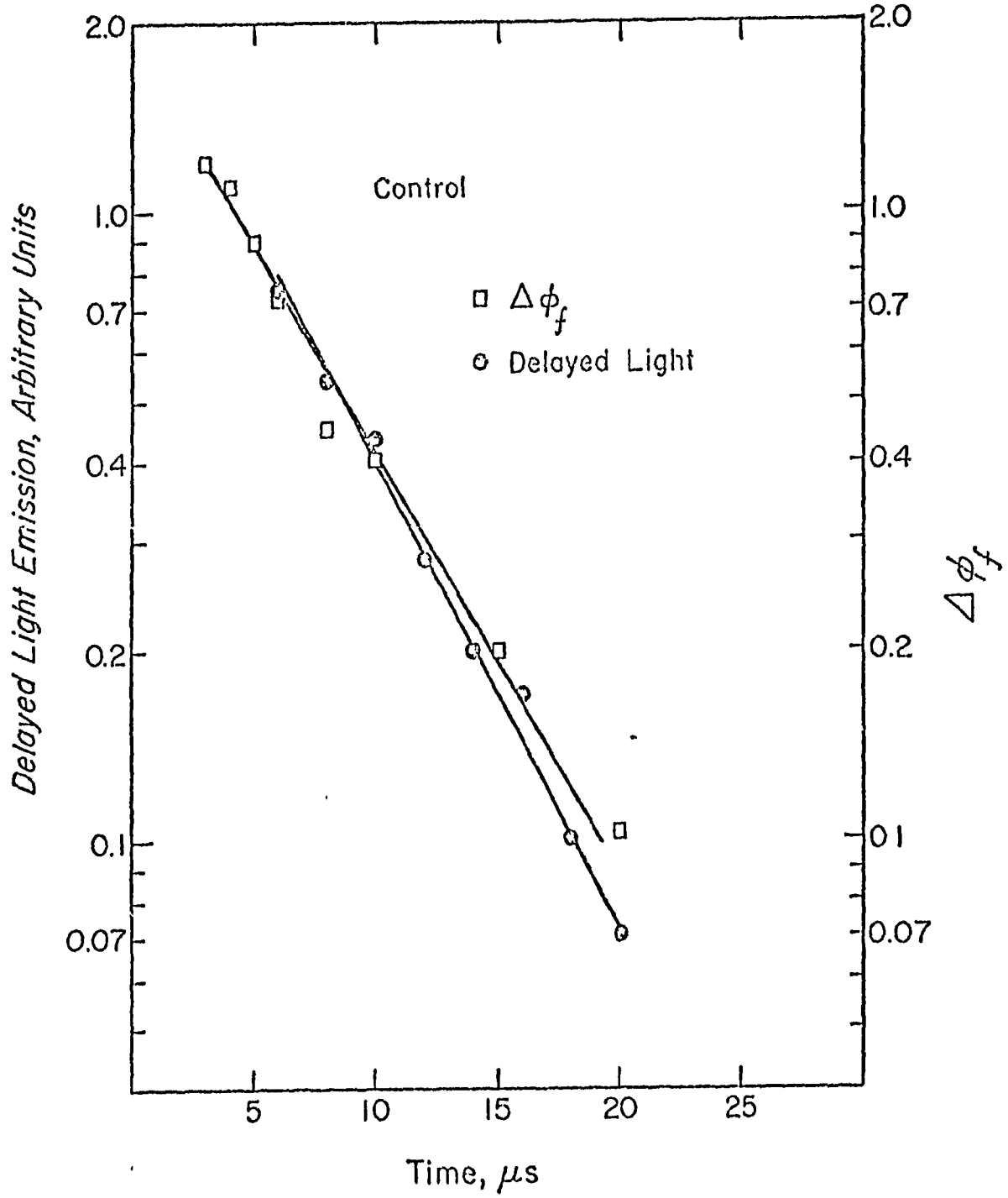
D. Discussion

1. Hydroxylamine Treatment in the Light and the ZP^+_{680} to Z^+P_{680} Reaction

The measurement of both the rise chlorophyll fluorescence yield and delayed light emission decay in the same sample allows a better understanding of the relationship between charge stabilization steps and delayed light emission decay. The rise in fluorescence yield in the microsecond time range has been associated [15, 66] with the following (*) charge stabilization reaction: $ZP_{680}Q \xrightarrow{h\nu} ZP^+_{680}Q^- \xrightarrow{(*)} Z^+P_{680}Q^-$, where Z is an electron donor to the Photosystem II reaction center chlorophyll P_{680} and Q is the "primary" electron acceptor. The fluorescence yield rises as P^+_{680}, which acts as a quencher of fluorescence, is converted to P_{680}. In normal chloroplasts, this reaction is found to be of first order with the rise in fluorescence yield being exponential, having a lifetime of 6 μs (Fig. 16). Fig. 23 is a replot of delayed light emission and fluorescence yield data which

Fig. 23. A plot of the most rapidly decaying delayed light component and the chlorophyll a fluorescence yield rise in the 3 to 20 μs range.

Logarithmic plot of the most rapidly decaying component of delayed light emission beginning at 6 μs after the excitation flash and the variable yield of chlorophyll a fluorescence yield, $\phi_f = \phi(\max) - \phi_f(t)$, beginning at 3 μs. $\phi_f(\max)$ is the maximum value of fluorescence yield and $\phi_f(t)$ is the value at some time t after the excitation flash. The delayed light emission decay component was obtained from decay curves by subtracting the contribution from slower decay components, and ϕ_f was obtained from fluorescence rise curves. All data are from pea chloroplasts at a chlorophyll concentration of 5 μg/ml receiving single flash excitation following 5 min or longer dark adaptation. $\Delta\phi_f$ is given in terms of ϕ_0, the yield of fluorescence prior to an excitation flash, and delayed light emission is in arbitrary units, but normalized to have the same value as $\Delta\phi_f$ at 6 μs.


Control
□ Δφf
o Delayed Light
Delayed Light Emission, Arbitrary Units
Δφf
Time, μs
2.0
1.0
0.7
0.4
0.2
0.1
0.07
5
10
15
20
25

emphasizes the exponential character of the delayed light emission decay and fluorescence yield rise and the parallelism between the two phenomenon with the same 6 μs lifetime component.

Chloroplasts treated with 2 mM NH_2OH in the light have no observable fluorescence yield rise (Fig. 16) due to complete or very efficient blockage of the $ZP^+_{680}Q^- \longrightarrow Z^+P_{680}Q^-$ reaction. This blockage must be very close to P^+_{680} as a number of preillumination flashes would have been required before any NH_2OH effect could be observed, as was the case for TRIS washing (Chapter III). The delayed light emission has a prominent 6 μs lifetime decay component which is greatly reduced in samples treated with 2 mM NH_2OH in the light. Thus, the 6 μs decay component exhibited by microsecond delayed light emission is a consequence of P^+_{680} stabilization by an electron donated from Z versus back reaction of P^+_{680} with Q^- which leads to light emission. As expected, when the charge stabilization step is eliminated by NH_2OH treatment charge recombination becomes more probable and the yield of delayed light emission (Table 4, see area values) shows a significant increase.

2. Comparison of the ZP^+_{680} to Z^+P_{680} Reaction in Chloroplasts to that in Chlorella

A previous measurement [49] indicated a 1 μs lifetime rise in chlorophyll *a* fluorescence yield; therefore, the $ZP^+_{680} \longrightarrow Z^+P_{680}$ reaction is suggested to proceed at a rate of 1 μs instead of 6 μs, which we observe. However, this 1 μs reaction time was observed under conditions different than ours: (1) samples were intact algal cells instead of chloroplasts; (2) excitation was provided by a just saturating xenon flash having a width at one-half height of 1 μs rather than a saturating N_2-laser pulse

with 10 ns width; and (3) the 1 μs rise was only observed on the first flash following 3 min dark adaptation while our 6 μs rise was seen following the first and succeeding flashes. The exact reason for this difference in this reaction lifetime is not known; nevertheless in our chloroplasts, following a saturating 10 ns flash, a 6 μs lifetime exponential rise in chlorophyll a fluorescence is observed (Figs. 16 and 23).

As discussed above for chloroplasts, we find a 6 μs delayed light emission decay component which correlates well with the 6 μs $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ reaction time from fluorescence yield rise measurements. Recently [65], a 0.7 to 1.4 μs delayed light emission component has been observed in Chlorella following one or more saturating laser flashes. The 0.7 to 1.4 μs delayed light emission decay time was also presumed to be caused by the disappearance of P^{+}_{680} by the $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ reaction believed to have a 1 μs reaction time [49]. No explanation was given as to why a 0.7 to 1.4 μs delayed light emission decay should be observed after flashes other than the first when $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ is known to not have a 1 μs reaction time [49]. We have also observed a 0.8 to 1.9 μs lifetime delayed light emission decay component in chloroplasts, but, as discussed in Chapter II, we conclude that this 0.8 to 1.9 μs transient signal from chloroplasts is an artifactual effect of the sample fluorescence rather than a 0.8 to 1.9 μs delayed light emission component. We wonder whether the 0.7 to 1.4 μs component observed in delayed light of Chlorella [65] is also a similar artifact since several conditions which give high fluorescence yield also give high delayed light emission; no definite conclusion can be drawn since a control of chlorophyll in solution, that we used, was not included in the recent work [65].

3. The Effect of Electron Donors on Chloroplasts Treated with NH_2OH in the Light

The inability of Mn^{2+} to reverse the NH_2OH effects on fluorescence yield rise and delayed light emission (Table 4, line 3) is not consistent with the suggestion [55] that Mn^{2+} donates electrons directly to P^+_{680}. The indirect electron flow from Mn^{2+} to P^+_{680} via Z was also suggested by fluorescence yield and delayed light measurements made on TRIS treated chloroplasts (Chapter III; also see ref. 74). It appears from the present data that Mn^{2+} donates electrons to Z, the first donor to P^+_{680}, and is subject to the block caused by NH_2OH treatment.

The inability of diphenyl carbazide to restore the fluorescence yield rise in chloroplasts treated with NH_2OH in the light (see Table 4, line 6) indicates that the site of its donation is also to Z and not directly to P^+_{680}. An antiserum prepared against a 11,000 molecular weight polypeptide by Schmid et al. [75] blocks electron donation by tetramethyl benzidine but not by diphenyl carbazide. The action of diphenyl carbazide was not understood well enough to say if the antiserum affected the Photosystem II reaction center directly or some secondary donor site on the oxygen side The latter interpretation for the site of the antiserum effect now seems most probable in view of the above information that diphenyl carbazide does not donate electrons directly to P^+_{680}, but, rather, to Z. Thus, it is likely that the 11,000 molecular weight polypeptide could have a significant relation to Z.

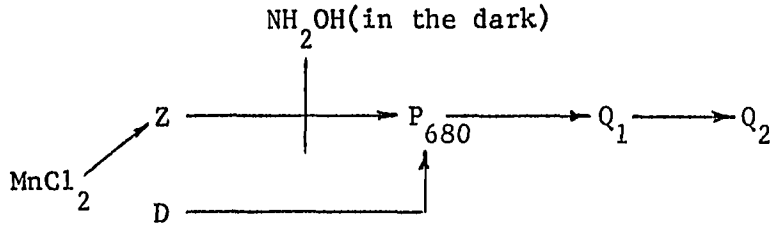
4. Hydroxylamine Treatment in the Dark and the ZP^+_{680} to Z^+P_{680} Reaction

As reported earlier in intact cells of Chlorella [15, 67], we also observed that treatment with millimolar concentrations of NH_2OH in the dark

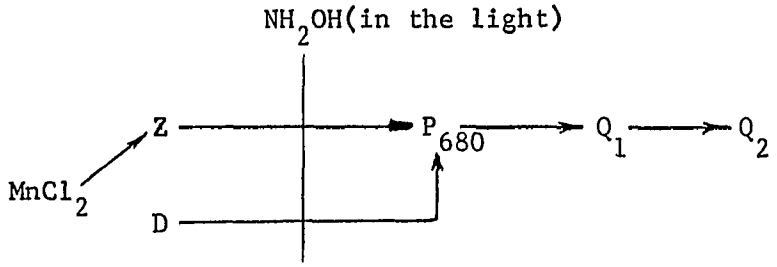
produces only a partial block of electron donation to P^+_{680} in isolated pea chloroplasts (Fig. 16). Electron donation to P^+_{680} continues after NH_2OH is washed out and the rise in chlorophyll a fluorescence yield shows no flash number dependents in our chloroplast samples. Contrary to this, the electron donation rate decreases after the first two flashes in Chlorella [67]. In chloroplasts, addition of 1 mM NH_2OH increases the rate of electron donation as judged by chlorophyll a fluorescence yield rise. The results with chloroplasts, as with Chlorella [15, 67], imply that some other donor (D) of electrons exists and donates electrons to P^+_{680} when NH_2OH has been washed out. Whether it acts as an intermediate electron carrier when NH_2OH is present is uncertain. In any case, electron donation to P^+_{680} continues, only at a slow rate, in chloroplasts treated with 2 mM NH_2OH in the dark.

5. The 50 to 60 μs Delayed Light Emission Decay Component

Chloroplasts treated with millimolar concentration of NH_2OH in the light and dark have an enhanced delayed light component with a 50 to 60 μs lifetime (Figs. 15 and 17 and Table 4 lines 2 and 7). Since for chloroplasts treated with NH_2OH in the light reduction of P^+_{680} by Z does not occur to an observable extent (Fig. 16), the 50 to 60 μs delayed light decay component must be associated with some other stabilization step in the reaction center. This 50 to 60 μs decay component cannot be the fundamental back reaction, $P^+_{680}Q^- \rightarrow P_{680} + h\nu_{DLE}$, since we would only expect to observe this component in samples having NH_2OH and DCMU present; however, slower decay components exist [32, 47] in the hundreds of microsecond time range. One possible explanation for the 50 to 60 μs component is that a charge stabilization, with this reaction rate, occurs on the acceptor side of Photosystem II. An electron flow diagram for Photosystem II can be written as follows:



and



where Q_1 and Q_2 are different states of the Photosystem II acceptor and the 50 to 60 μs delayed light emission component is correlated with the rate of change from Q_1 to Q_2. Q_1 and Q_2 could be separate charge carriers but it would be more acceptable to current views of the electron capacity of the primary acceptor if they were merely different states of a single component. The existence of heterogeneity in the Photosystem II acceptor has been suggested earlier from redox titration data [76] and chlorophyll a fluorescence yield data [77, 78].

6. Microsecond Delayed Light Emission Flash Number Dependence With and Without NH_2OH

The peak in the microsecond delayed light emission flash pattern being delayed to the sixth flash as was the oxygen yield pattern for these samples, by the presence of 50 mM NH_2OH, suggests the charge accumulation in the oxygen evolving system modulates the yield of delayed light also in the microsecond time range. The delay in the oxygen yield peak has been

suggested to be caused by two or three NH_2OH molecules at each reaction center donating electrons to the oxygen evolving system charge accumulator [70]. These low concentrations of NH_2OH have no effect on the $ZP^+_{680}Q^-$ $Z^+P_{680}Q^-$ reaction since the chlorophyll a fluorescence yield rise and delayed light emission decay are unchanged (Fig. 12). In control chloroplasts, the second flash gave essentially zero oxygen yield while delayed light emission is quite high (Figs. 13 and 14). This behavior of high delayed light yield after the second flash has been reported previously by Duysens et al. [49] in Chlorella for a delayed light emission component with a 20 μs lifetime. Zankel [59] observed that the intensity of delayed light emission at 90 μs after a flash in spinach chloroplasts followed the oxygen yield pattern quite closely, being low on the second flash and maximum on the third. The reason for this discrepancy in results is unknown. A high value of delayed light emission is expected after the second flash since other charged states of the oxygen evolving system besides S_4 are involved in delayed light emission [17, 24]. The oscillations in microsecond delayed light emission follow the oscillations in the concentration of $S_2 + S_3$ [79]. The damping of the oscillations in delayed light emission is seen to be much more rapid than for oxygen yield (Figs. 13 and 14) and is believed to occur because delayed light emission is affected to a different extent by each charge state of the oxygen evolving system [17].

7. Changes in Microsecond Delayed Light Emission Caused by Altering the Concentration of P^+_{680} and Q^-

If microsecond delayed light emission is due to P^+_{680} and Q^- recombination, then conditions which cause the Photosystem II reaction center to be in a closed state, such as $P_{680}Q^-$, or cause Q^- to become oxidized,

should result in an elimination of delayed light emission. Three different chloroplast treatments were used to accomplish this:

(1) The $P_{680}Q^-$ state was brought about by the addition of DCMU to block Q^- decay by electron flow to the intersystem electron carried pool (A) and by giving a second flash rapidly enough so that Q^- oxidation by any other pathyway(s) is very small. The reduction of P^+_{680} in control chloroplasts is complete within about 20 μs (Fig. 16). Even with a second flash given as rapidly as 133 ms after the first flash, approximately 70% of the delayed light emission remains and the 30% drop in delayed light emission disappears at approximately the same rate as Q^- becomes reoxidized (Fig. 20). This agrees well with earlier results [59]; however, a residual amount of delayed light emission with a rapid second flash was not reported. (This will be discussed later.) On the third and succeeding flashes, the level of delayed light emission remains the same as after the second flash.

(2) In NH_2OH treated samples, the reaction centers could be put in the closed form, $P_{680}Q^-$, by having DCMU present and preilluminating the sample. The rate at which centers become closed, of course, depends upon the tendency for electrons to pass through the NH_2OH block from Z to P^+_{680}. The completeness of the NH_2OH block is reflected in the time taken for reaction centers to become closed as indicated by inhibition of delayed light emission. As expected from fluorescence yield data (Fig. 16), NH_2OH treatment in the light gave the most complete block followed by NH_2OH treatment in the dark without and then with NH_2OH present (see Fig. 19). A residual amount of delayed light emission is found even after long periods of preillumination which puts all reaction centers in the closed state $P_{680}Q^-$. The properties of this residual delayed light emission including intensity and decay rate

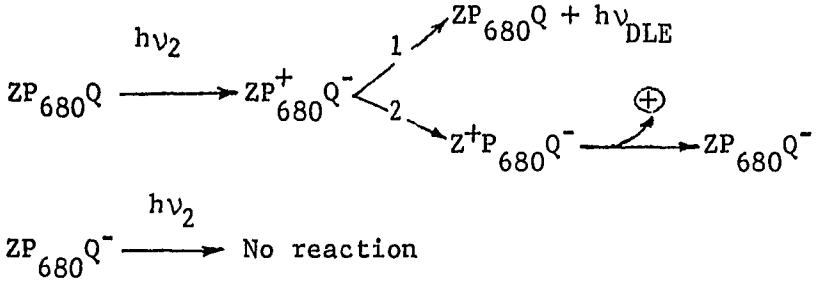
(Fig. 18) are identical to those of the residual delayed light emission observed in DCMU treated control samples.

(3) Silicomolybdate was used to accept charge directly from Q^- and, thus, it competes with P^+_{680} recombination for the Q^- charge. Delayed light emission is greatly inhibited when silicomolybdate is present (Fig. 21) in chloroplasts that have active Photosystem II reaction centers, as demonstrated by their ability to evolve oxygen. The small amount of delayed light remaining when silicomolybdate is present (Fig. 21) is probably due to the incomplete acceptance of charge from Q^- by silicomolybdate on the microsecond scale.

All three of the above treatments cause a decrease in microsecond delayed light emission in accordance with the charge recombination theory. However, the first two treatments have a residual delayed light emission indicating that these treatments did not cause all reaction centers to become closed. The following explanation is given for this results. In samples having just DCMU present, the following reactions take place on the first flash ($h\nu_1$):

$$ZP_{680}Q \xrightarrow{h\nu_1} ZP^+_{680}Q^- \begin{cases} \xrightarrow{1} ZP_{680}Q + h\nu_{DLE} \\ \xrightarrow{2} Z^+P_{680}Q^- \xrightarrow{\oplus} ZP_{680}Q^- \end{cases}$$

where reaction 1 is the charge recombination leading to delayed light emission and open ($ZP_{680}Q$) reaction centers and reaction 2 is charge stabilization giving closed ($ZP_{680}Q^-$) reaction centers. After a second flash ($h\nu_2$) the following reactions take place:



and a population of open and closed reaction centers will exist. We suggest, however, that reaction 1 occurs much more readily after the second flash due to the presence of centers in the $ZP_{680}Q^-$ form prior to the second excition so that a significant delayed light emission level is observed in spite of the low concentration of active centers ($ZP_{680}Q$). (For an earlier proposal of the influence of one reaction center on another, see Mar and Roy [80].) Thus, an equilibrium will be established with both open and closed reaction centers present, and residual delayed light emission will occur. The same type of reasoning will explain the occurrence of residual delayed light emission in NH_2OH treated chloroplasts with DCMU present.

The possibility that Q_1 and Q_2, proposed earlier in this chapter to be different states of the acceptor, are separate charge carriers can also account for the existence of delayed light emission on the second flash in samples with DCMU present. The existence of a carrier (W) prior to the classical Q has recently been proposed by Van Best and Duysens [65] based on different reasons. However, neither proposal accounts for the similarity of the decay kinetics between control plus DCMU and preilluminated NH_2OH treated samples plus DCMU (Fig. 18). A more comprehensive model must be developed upon further research.

8. Quantum Yield of Delayed Light Emission

The quantum yield of delayed light emission (ϕ_{DLE}) of 3.1×10^{-4} for decay components beginning at 6 μs after the flash, as expected, is larger

than the 10^{-4} value Zankel [59] obtained for only the 50 μs lifetime decay component. (As already noted, in Chlorella, a 1 μs delayed light emission component has been reported [65]. If we normalize the aerobic Chlorella delayed light emission signal to that from chloroplasts at 10 μs, then we find the ϕ_{DLE} contributed by the 1 μs component extrapolated to zero time is 9×10^{-5}. Thus, the total ϕ_{DLE}, if chloroplasts are presumed to have a 1 μs component as do Chlorella, is about 4×10^{-4}.) Ruby [81] measured delayed light emission in the 1-700 ms time range after a single flash in Chlorella and found ϕ_{DLE} for millisecond delayed light emission to be 10^{-5}. Similar measurements after a single flash in the 1.5 ms to 30 s time range with spinach chloroplasts [82] gave a value of 4×10^{-7} for ϕ_{DLE}. Using the technique of modulating delayed light emission with an electric field [83], the ϕ_{DLE} in chloroplasts for the time range 1 s to 1 min was approximately 10^{-4} [84]. Since this measurement of ϕ_{DLE} was made during continuous illumination and only a portion of the delayed light emission is modulated by the applied electric field [83], a comparison of the value of ϕ_{DLE} to those obtained by other methods has little meaning. Our value for ϕ_{DLE} in samples treated with NH_2OH in the light and having DCMU present (but not preilluminated), so that all charge stabilization reactions have been inhibited, is only about 4% of the fluorescence yield. This indicates that under conditions which will maximize the charge recombination back reaction, over 90% of the recombinational energy is unobserved. This may be only partly (see above) due to delayed light emission decay components with lifetimes smaller than 6 μs at times $t < 6$ μs [49] which we do not measure but mainly due to a large portion of the recombinational energy being dissipated by nonradiative pathways. Since components with lifetimes smaller than 6 μs do not exist with NH_2OH present [65], the latter suggestion is favored.

CHAPTER V

MEMBRANE POTENTIAL AND MICROSECOND TO MILLISECOND DELAYED LIGHT EMISSION

A. Introduction

Delayed light emission is a radiative decay route for metastable states formed during illumination of photosynthetic organisms and is sensitive to treatments that alter photosynthetic reactions. Modifications of the high energy state of phosphorylation by uncoupling agents [85] and production of transmembrane pH and salt gradients [86, 87, 88, 89] strongly affect the intensity of delayed light emission in the millisecond and seconds time range. During illumination the induction kinetics of millisecond delayed light emission are controlled not only by electron transport but also by the development of the light induced proton uptake and membrane potential [89-91]. These effects were ascribed to a modulation of the effective activation energy for emission by the electrical and chemical gradients of the protomotive force of the chemiosmotic coupling hypothesis [92, 93].

The effect of salt-jump or light induced membrane potential [89, 90] was established only for samples that had been preilluminated with continuous light or multiple pulses associated with the phosphoroscope method. Also, the present theory of high energy state enhancement of delayed light emission does not differentiate between delayed light at different times, which may arise from charge recombination in the Photosystem II reaction center in different stabilization states [17].

All earlier studies, noted above, had used techniques, which generate a proton gradient during preillumination. There is an indication [94] that

a proton gradient may be required to observe membrane potential effects on delayed light emission in the seconds range. We report here the effects of salt-induced and light-generated membrane potential on both the microsecond and millisecond delayed light emission after a single saturating 10 ns laser flash. The use of single flash excitation allowed us to determine if a proton gradient is required to observe membrane potential effects.

B. Materials and Methods

In these experiments chloroplasts from Alaska pea leaves were used. Chloroplast extraction procedures are described in Chapter II. For salt-jump experiments chloroplasts were treated as described in ref. [94]; they were washed twice and finally resuspended in a medium containing 5 mM N-tris(hydroxymethyl)-methyl-2aminoethanesulphonic acid (TES) and 0.4 M sucrose (adjusted to a pH of 7.1 with KOH; the final concentration of KOH was 2 mM).

The absorption changes at 518 nm were recorded with a conventional single beam spectrophotometer having an electronic risetime of 10 μs. Single saturating flash excitation (pulse width at half height, 300 ns) was provided by a Phase-R Model DL-110 V dye laser operated at 660 nm with the following dye mixture: 80 μM cresol violet perchlorate and 50 μM rhodamine 6-G in methanol. The photomultiplier was protected from the actinic flash with a Corning CS 4-96 glass filter and the output signal was displayed on a Tektronix storage oscilloscope and photographed.

Light induced pH changes were measured with a Beckman 39505 Futura Combination Electrode and the output from a Beckman Phasar-I digital pH meter was displayed on a chart recorder. Illumination from a slide projection

lamp was passed through a 2 inch water filter, a lens, and a Kodak Wratten #16 orange gelatin filter; the incident light intensity at the sample was 0.13 watts/cm^2.

The apparatus for measuring delayed light emission in the microsecond and millisecond ranges has been described in Chapter II. Preillumination was provided by the laser operating in a rapid pulse mode at a frequency of 32 Hz. For delayed light emission measurements in the seconds time range, continuous broad-band blue light, obtained from an incandescent lamp and passed through water and Corning CS 4-96 glass filters, was used; the intensity of this light incident at the sample was 10 mW/cm^2.

C. Results

1. Effects of Gramicidin D on Microsecond Delayed Light Emission

Flash illumination of chloroplasts produces a light generated membrane potential, ψ_1, [95] across the thylakoid membrane. In order to demonstrate the ψ_1 was being generated in our chloroplasts (50 µg chlorophyll/ml), the electric field indicating absorption change at 518 nm was measured beginning at 25 µs after an excitation flash (Fig. 24). The amplitude and decay time are in agreement with a range of reported results [95]. According to estimates made for chloroplasts [95], based on thylakoid membrane capacitance and approximate displaced charge, this absorption change corresponds to a light generated potential of 50 to 100 mV. Addition of 1 µM gramicidin D (1 gramicidin D per 50 chlorophyll molecules) completely eliminated the 518 nm absorption change, and thus ψ_1, in our samples in the microsecond time range. Therefore, the microsecond delayed light emission was expected to be affected by gramicidin D in the same manner postulated to occur for millisecond delayed light.

Fig. 24. Absorption changes at 518 nm from 25 μs to 60 ms after an excitation flash.

Logarithmic plot of $\Delta I/I$ (I is the transmitted light intensity) at 518 nm from 25 μs to 60 ms after a 660 nm flash. The chlorophyll concentration was 50 μg/ml and the optical pathlength was 1 cm. The chloroplasts were suspended in the following medium: 50 mM sodium phosphate, 400 mM sucrose and 40 mM KCl at pH = 7.8.

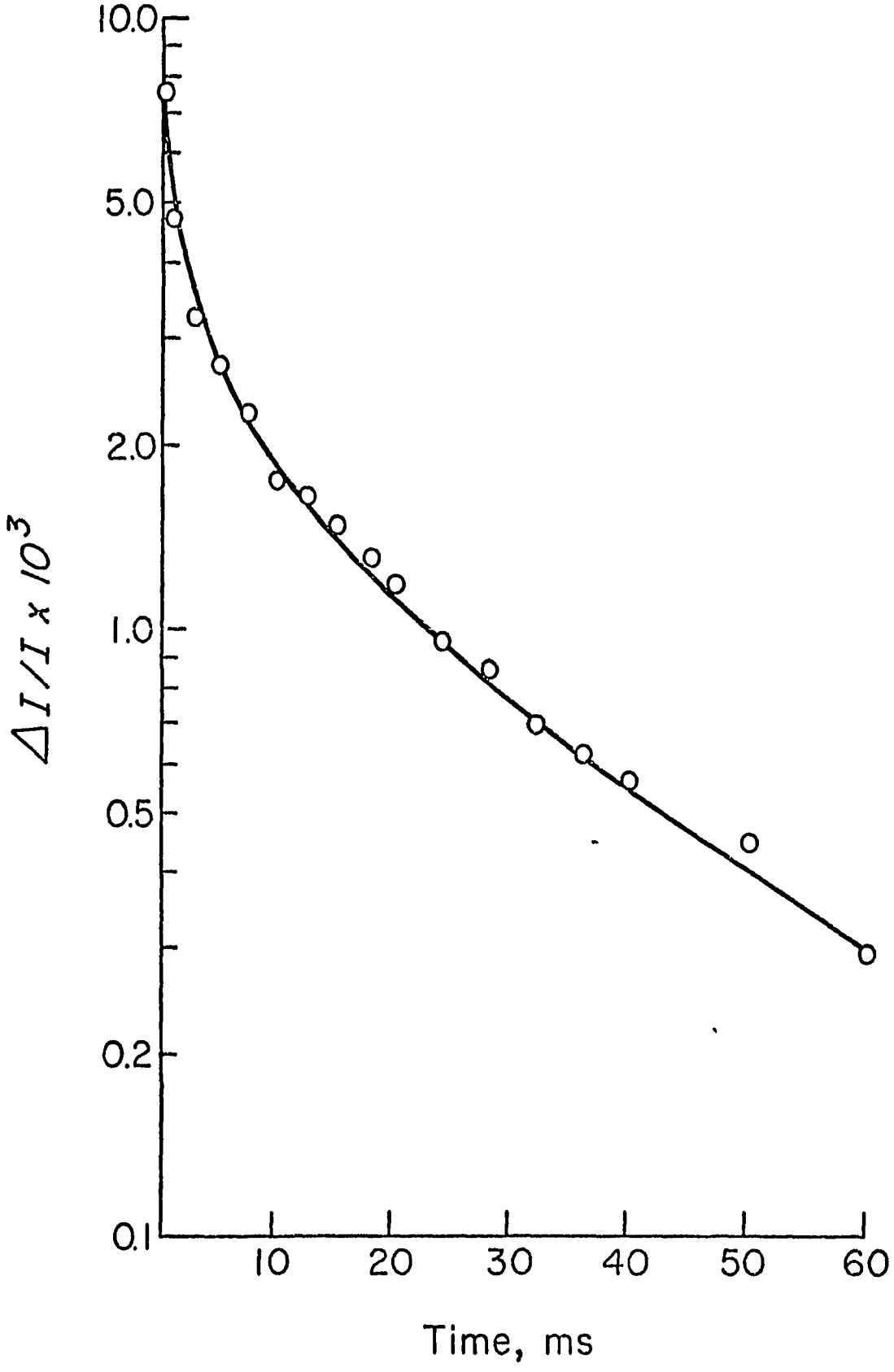

10.0
5.0
2.0
1.0
0.5
0.2
0.1
ΔI/I x 10³
10
20
30
40
50
60
Time, ms

Fig. 25 shows delayed light emission decays from the same chloroplasts as used above but 5 μg chlorophyll/ml, beginning 6 μs after a flash, with and without gramicidin D (up to 1 gramicidin per 5 chlorophyll molecules). Complications due to changes in the electron flow rate from Q^-, the reduced form of the "primary" acceptor of Photosystem II, to the intersystem carrier are not expected since these decays are after a single flash. Gramicidin D had no effect on microsecond delayed light emission decay after the first flash even at ten times the concentration per chlorophyll needed to eliminate the 518 nm light induced absorption change (Fig. 25).

An effect of 1 μM gramicidin D (1:5 chlorophyll molecules) on microsecond delayed light emission was observed (Fig. 26) only with chloroplasts that had received at least ten preillumination flashes (given at a rate of 1 flash/2s). However, 0.1 μM gramicidin D caused very little or no change in microsecond delayed light emission even though this concentration was sufficient to completely eliminate the 518 nm absorption change (Fig. 26) in this time range. Addition of 0.1 M 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU) (DCMU to chlorophyll = 1 to 50) inhibited microsecond delayed light emission to the same extent as 1 μM gramicidin D and gramicidin had no additional effect. Thus, the effect of high concentration of gramicidin may be due to its secondary inhibitory effect on electron flow. This was supported by our observation that 1 μM gramicidin D causes a decrease in oxygen evolution in these chloroplasts.

2. Effects of Gramicidin D and Nigericin on Millisecond Delayed Light Emission

The membrane potential generated by a single flash persists well into the millisecond time range (Fig. 24). We looked for the effects of this

Fig. 25. Delayed light in the 6 to 100 μs range in dark-adapted chloroplasts with and without gramicidin D.

Logarithmic plot of delayed light emission in the 6 to 100 μs time range after a single saturating 10 ns 337 nm flash. Chloroplasts were dark adapted for 5 min prior to excitation. The chlorophyll concentration was 5 μg/ml, and the suspension medium was 50 mM phosphate, 400 mM sucrose, and 40 mM KCl at pH = 7.8. Sample conditions are; (o—o—o), control, and (■—■—■), plus 1 μM gramicidin (1 gramicidin:5 chlorophyll).

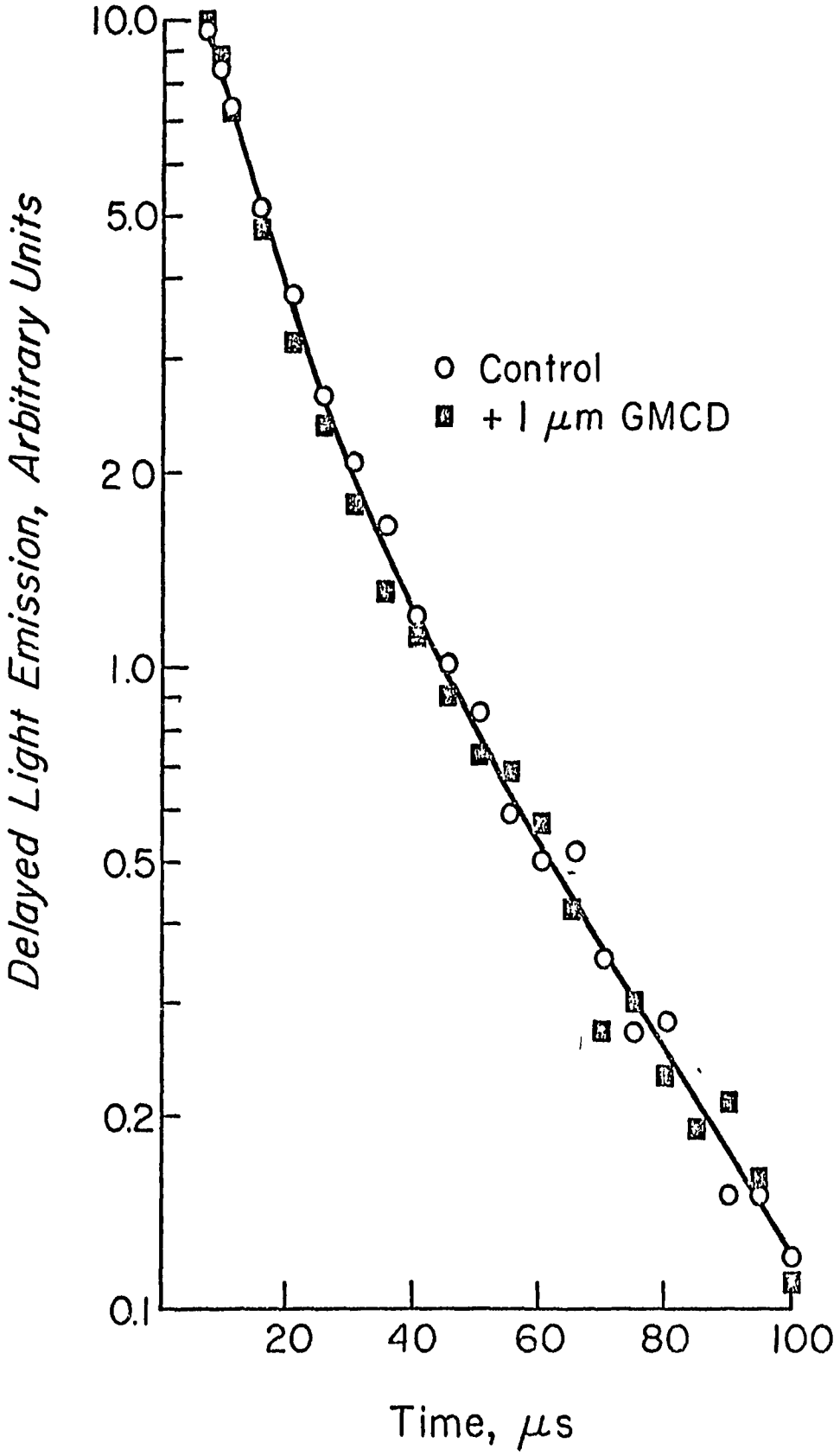
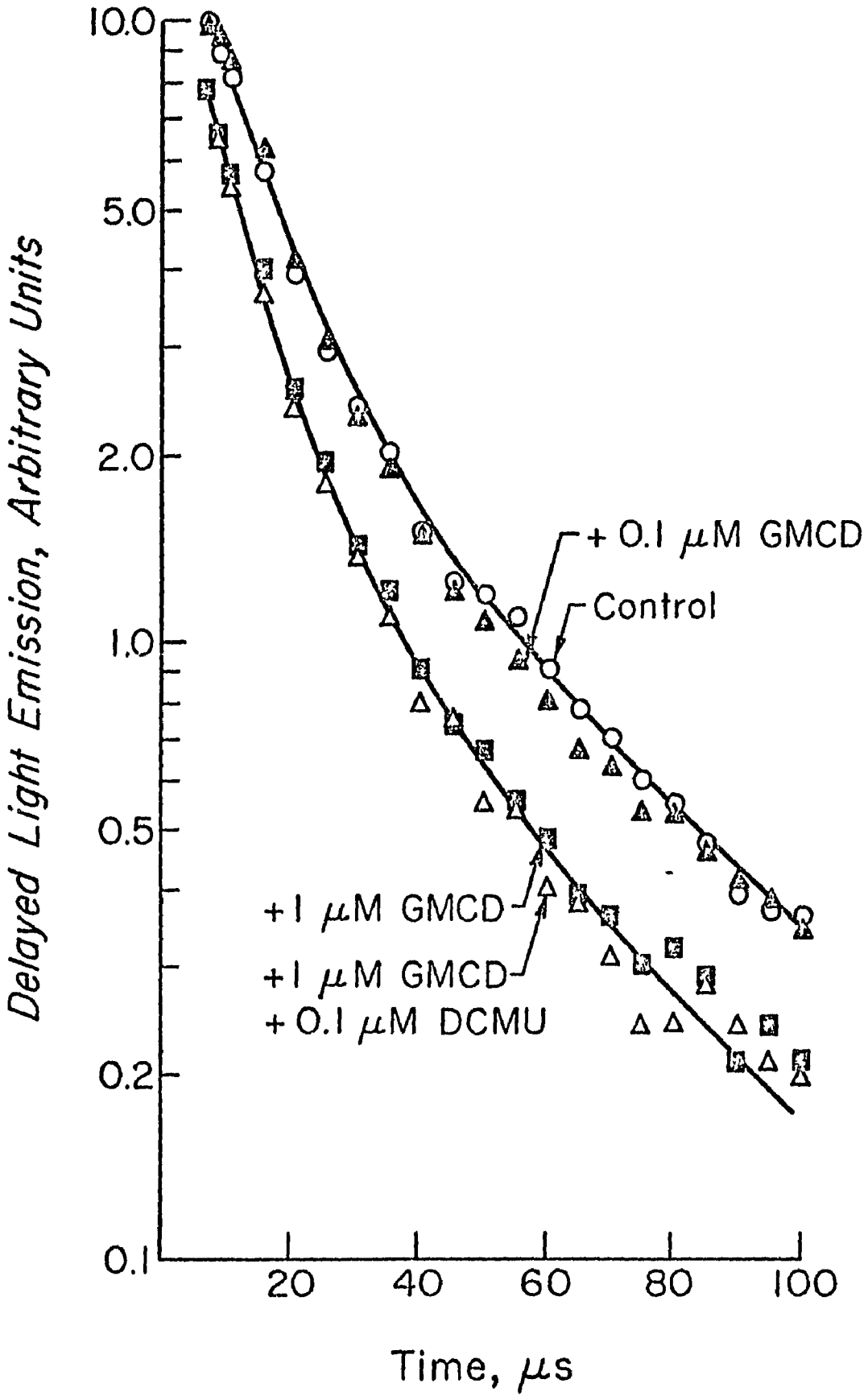

10.0
5.0
2.0
1.0
0.5
0.2
0.1
20
40
60
80
100
Delayed Light Emission, Arbitrary Units
Time, μs
Control
+ 1 μm GMCD

Fig. 26. Delayed light in the 6 to 100 μs range in preilluminated chloroplasts with and without gramicidin D.

Logarithmic plot of delayed light emission in the 6 to 100 μs range following seven or more preillumination flashes given at a rate of 1 flash/2s. The chlorophyll concentration was 5 μg/ml, and the suspension medium was 50 mM phosphate, 400 mM sucrose and 40 mM KCl at pH = 7.8. Sample conditions are: (o—o—o), control; (▲—▲—▲), plus 0.1 μM gramicidin (1 gramicidin:50 chlorophyll); (■—■—■), plus 1 μM gramicidin; and (△—△—△), plus 1 μM gramicidin and 0.1 μM DCMU (1 DCMU:50 chlorophyll).


10.0
5.0
2.0
1.0
0.5
0.2
0.1
20
40
60
80
100
Delayed Light Emission, Arbitrary Units
Time, μs
+ 0.1 μM GMCD
Control
+1 μM GMCD
+1 μM GMCD
+ 0.1 μM DCMU

membrane potential on millisecond delayed light emission after a single flash. As shown in Fig. 27 (a), 1 μM gramicidin D (1 gramicidin D per 5 chlorophylls) caused no change in the millisecond delayed light emission following a single excitation flash. A large gramicidin D effect was observed (Fig. 27 (b)) only if the sample had been preilluminated for 30 s with laser flashes given at a rate of 32 Hz. This inhibition of delayed light emission by gramicidin D confirms earlier results [85, 89]. The 518 nm absorption measurements showed that 0.1 μM gramicidin D entirely eliminated the light generated membrane potential in this time range; the inhibition of delayed light emission by gramicidin in Fig. 27 (b) could, therefore, be due to the loss of the light generated membrane potential.

An enhancement of delayed light emission by preillumination (Fig. 27) was optimal with 30 s of preillumination. To test whether this slow build up of delayed light emission was due to the involvement of an H^+ ion gradient across the thylakoid membrane, nigericin (an ionophore which inhibits net H^+ ion uptake [96]) was added; it eliminated the enhancement of delayed light emission by preillumination (Fig. 27 (c)) in agreement with previous results [90]. The addition of 1 μM valinomycin (an ionophore which specifically increases membrane permeability for K^+ [97]) only partially inhibited the preillumination enhancement of delayed light emission (Fig. 27), which is similar to the 0.1 μM gramicidin D effect (Fig. 27 (b)). At these concentrations it seems that gramicidin D and valinomycin eliminate the membrane potential enhancement of delayed light emission leaving the additional enhancement which can be suppressed by nigericin.

The enhancement of delayed light emission by preillumination was related to net H^+ uptake (Fig. 28). The decay of the intensity of delayed

Fig. 27. Delayed light in the 0.6 to 4 ms range in chloroplasts plus gramicidin, nigericin, or valinomycin.

Delayed light emission in the 0.6 ms to 4 ms range. The chlorophyll concentration was 5 μg/ml and the suspension medium was 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH). (a) After a single excitation flash with (– – –) or without (————) 0.1 μM gramicidin, (b) after a single excitation flash (————) and following 30 s of preillumination with laser flashes given at a rate of 32 Hz with (—— · ——) and without (– – –) 1 μM gramicidin, (c) 30 s of preillumination with laser flashes given at a rate of 32 Hz with (————) and without (– – –) 0.5 μM nigericin, and (d) with (————) or without (– – –) 1 μM valinomycin.

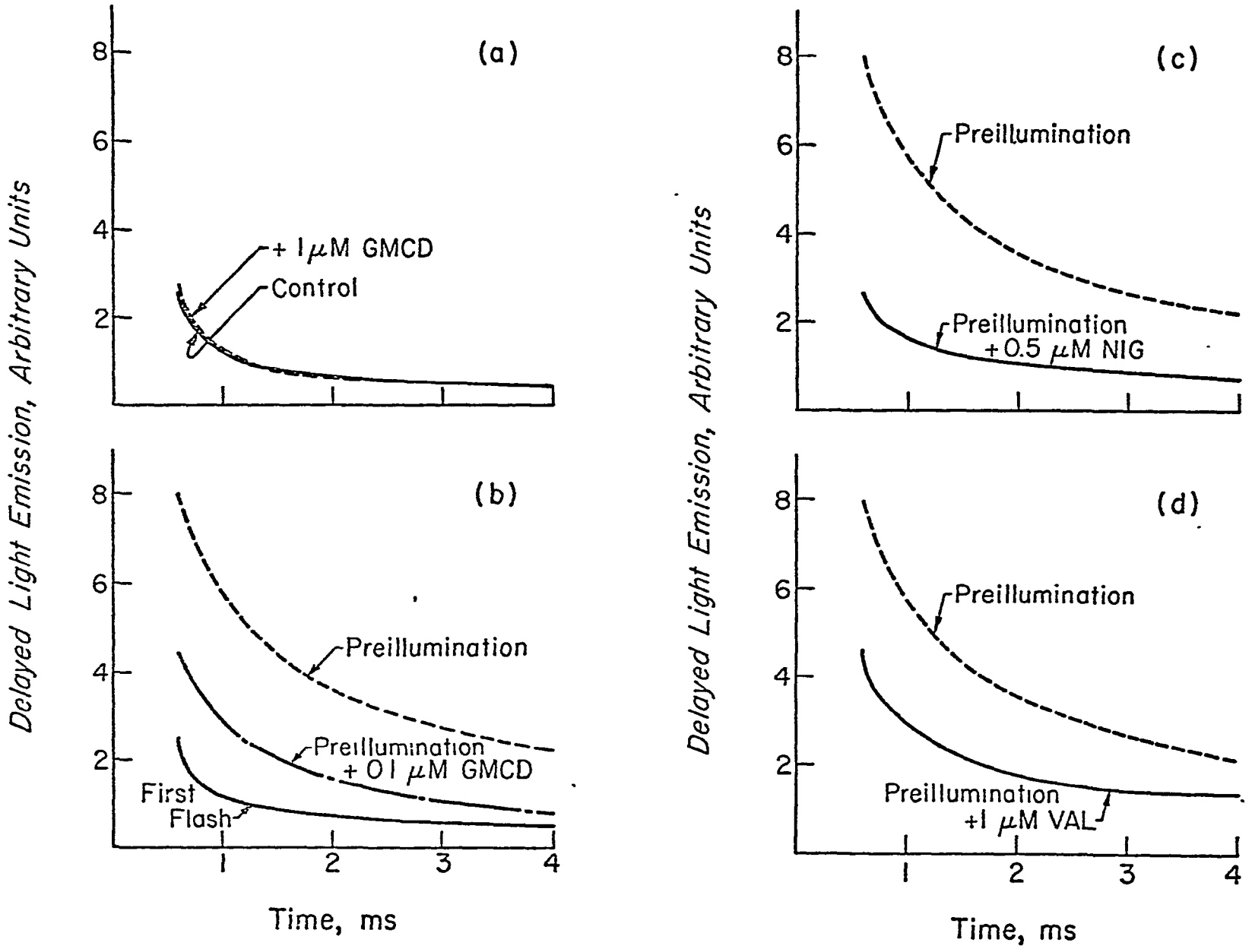
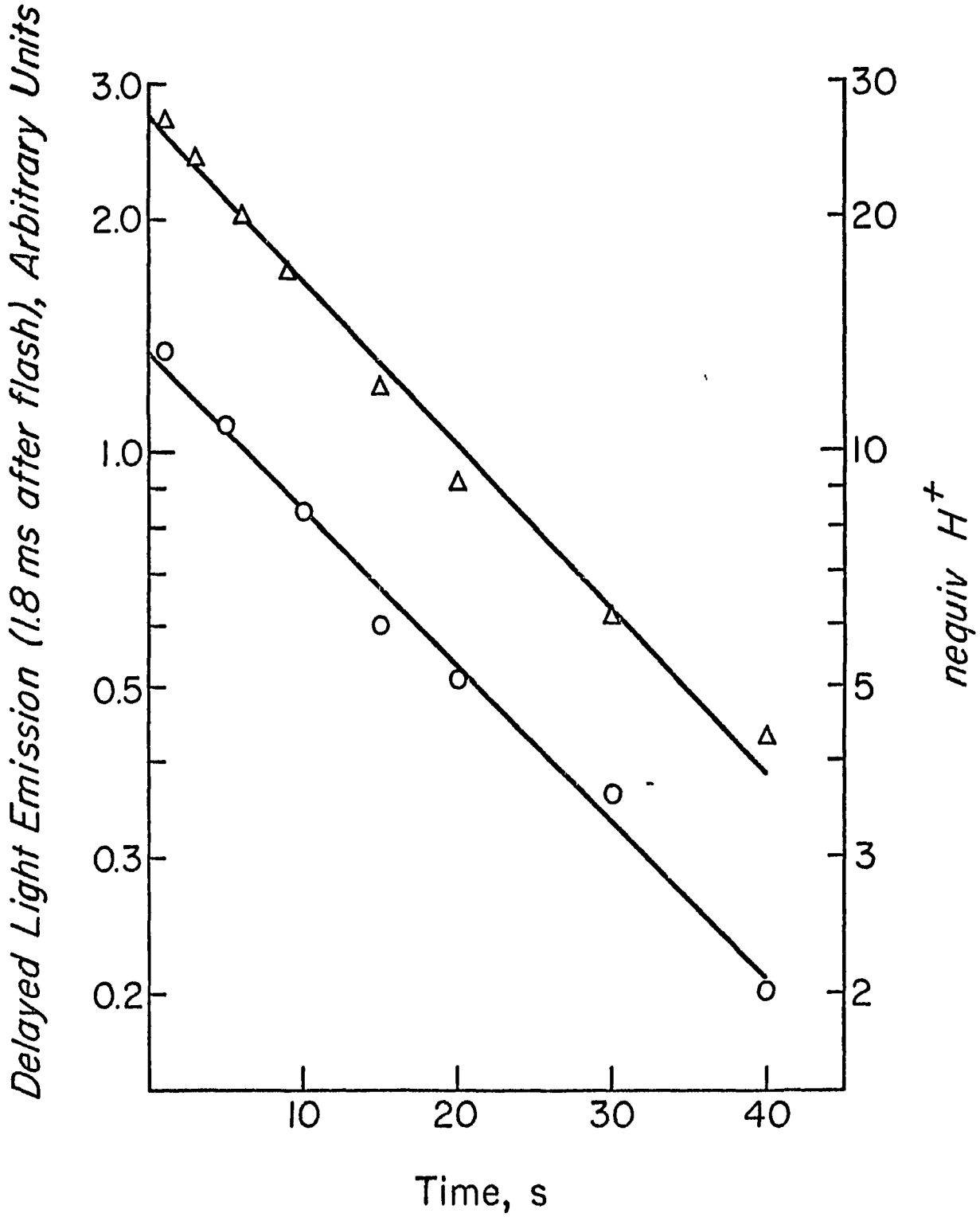

(a)
+ 1 μM GMCD
Control
(b)
Preillumination
Preillumination
+ 01 μM GMCD
First
Flash
(c)
Preillumination
Preillumination
+0.5 μM NIG
(d)
Preillumination
Preillumination
+1 μM VAL
Delayed Light Emission, Arbitrary Units
Time, ms

Fig. 28. Delayed light intensity at 1.8 ms and external H^+ ion concentration versus time after preillumination.

The lower curve (o—o—o) is a logarithmic plot of the delayed light emission intensity at 1.8 ms after a final excitation flash versus the time at which the final flash was given after the end of a 30 s preillumination period with laser flashes given at a rate of 32 Hz. The chlorophyll concentration was 5 μg/ml and the suspension medium was 400 mM sucrose, 40 mM KCl, and 50 mM sodium phosphate at pH = 7.8. The upper curve (Δ—Δ—Δ) is a logarithmic plot of the external H^+ ion concentration versus the time after termination of 30 s continuous illumination. The chlorophyll concentration was 25 μg/ml and the suspension medium was 40 mM KCl at pH = 7.8.


Delayed Light Emission (1.8 ms after flash), Arbitrary Units
3.0
2.0
1.0
0.5
0.3
0.2
30
20
10
5
3
2
nequiv H^+
10
20
30
40
Time, s

light emission at 1.8 ms after a single flash given at various times after termination of preillumination was kinetically identical to the decay of the light induced H^+ uptake.

3. Effects of Salt-Jump Induced Membrane Potential on Delayed Light

A transmembrane potential can be generated by means of ion diffusion gradients set up by the rapid injection of permeant salts [87, 88]. As noted earlier, these experiments dealt with such salt-jump effects on delayed light emission only after continuous illumination or with the phosphoroscope in the millisecond and seconds range. We measured the effects of the salt-jump on delayed light emission in the microsecond and millisecond range after a single flash.

An enhancement of delayed light emission generated by injection of 120 mM KCl to preilluminated chloroplasts in the presence of 1 μM valinomycin is shown in Fig. 29. (Injection of NaCl caused no enhancement of delayed light emission, data not shown.) This figure, which is in agreement with the results of Barber and Kraan [88], demonstrates that to see a maximal salt-jump effect, under single flash conditions, the excitation flash must be given approximately 0.5 s after the salt injection.

Millisecond delayed light emission, elicited by single flash excitation of dark adapted chloroplasts, was not enhanced by prior injection of KCl, but was enhanced by sodium benzoate (Fig. 30 (a) and (b)). Sodium benzoate establishes its own proton gradient across the thylakoid membrane. (See the Discussion for more detailed arguments.) The sodium benzoate induced enhancement of delayed light emission, after a single flash, was eliminated by 0.5 μM nigericin (Fig. 30 (c)). A KCl-jump enhancement of millisecond delayed light emission was observed only if the chloroplasts were preillumin-

Fig. 29. Delayed light emission in the seconds range with a salt(KCl)-jump.

Delayed light emission decay in the seconds range following termination of 10 s continuous illumination. The chlorophyll concentration was 25 μg/ml and the suspension medium was 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH) with 1 μM valinomycin present. A small volume of 3 M KCl was injected at two seconds after termination of illumination to obtain a final external KCl concentration of 120 mM.

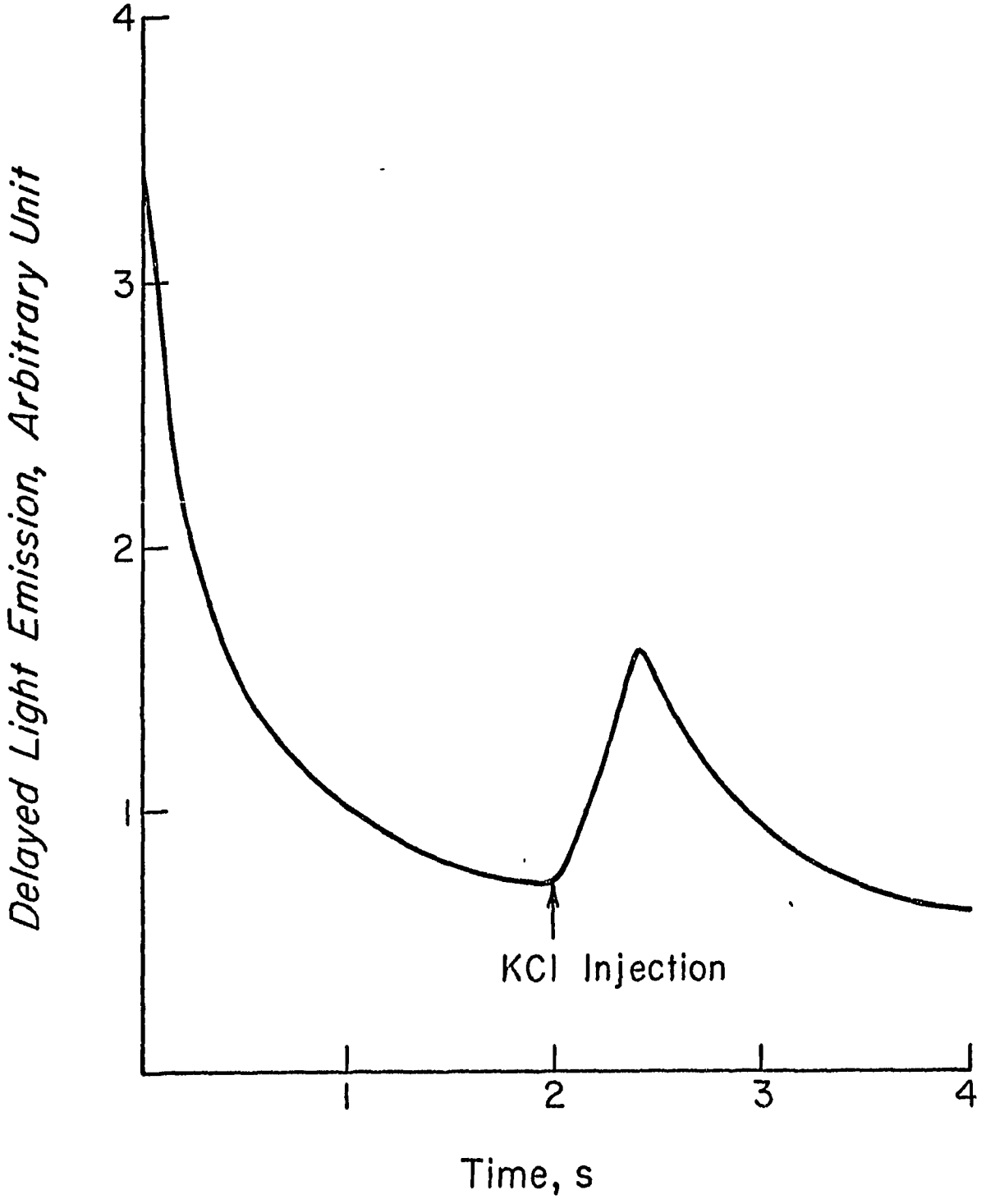
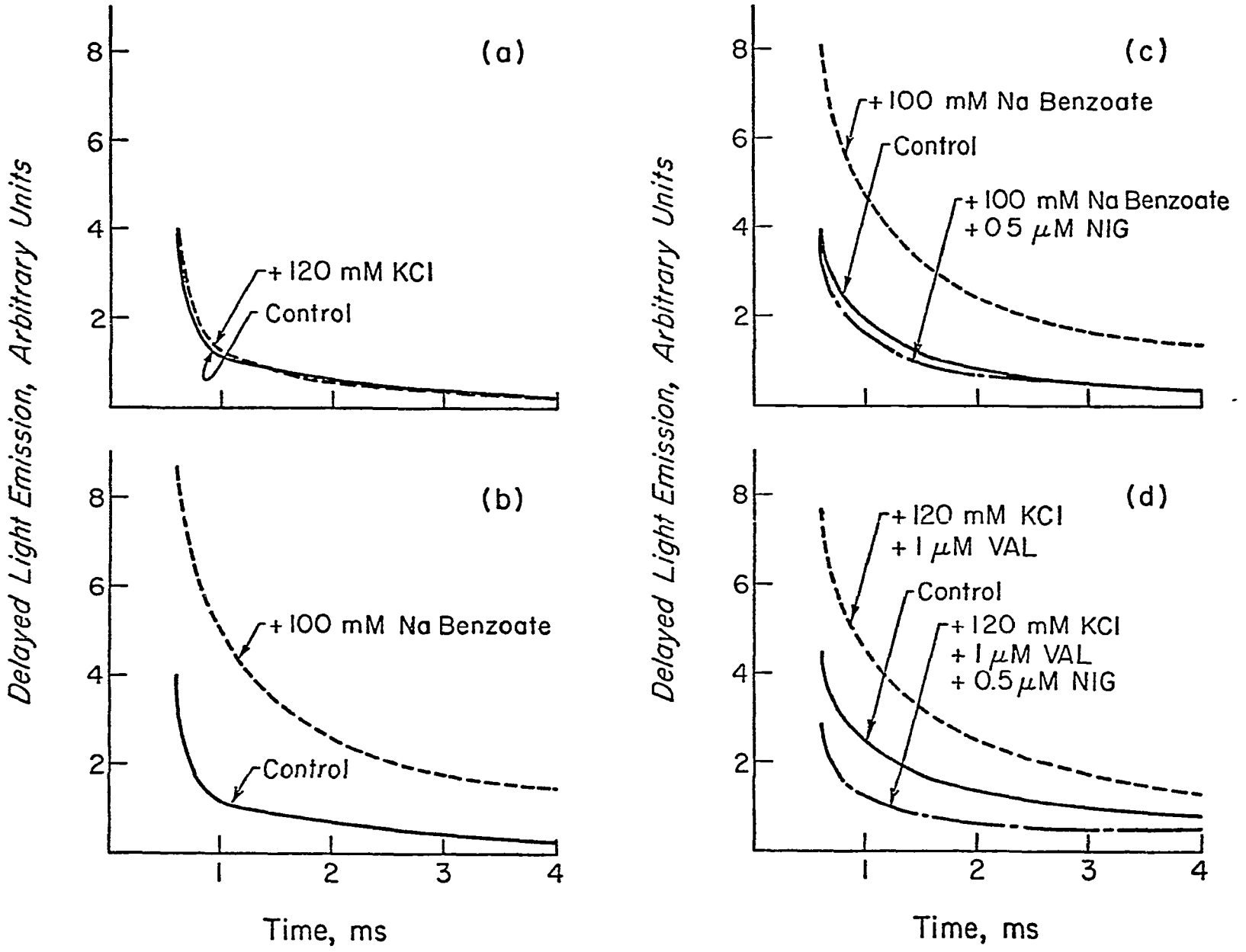

Delayed Light Emission, Arbitrary Unit
4
3
2
1
KCl Injection
1
2
3
4
Time, s

Fig. 30. Delayed light in the 0.6 to 4 ms range after various salt injections.

Delayed light emission decays after salt-jumps in the millisecond range. The chlorophyll concentration was 5 μg/ml and the suspension medium was 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH). Salt-jumps were made by injecting small volumes of 3 M salt solutions to obtain final concentrations as indicated in the figure. Except for the control (————), all decays are from a final laser flash given 0.5 s after a salt injection under the following conditions: (a) after a single flash preceeded by KCl injection (– – –) to a final concentration of 120 mM with 1 μM valinomycin present; (b) after a single flash preceeded by sodium benzoate injection (– – –) to a final concentration of 100 mM; (c) after a single flash preceeded by sodium benzoate injection to a final concentration of 100 mM with (— – —) or without (– – –) 0.5 μM nigericin present; and (d) decays in preilluminated samples after a final flash preceeded by KCl injection to a final concentration of 120 mM with 1 μM valinomycin (– – –) or 1 μM valimomycin and 0.5 μM nigericin present (— – —). Preillumination was 30 s of laser pulses given at a rate of 32 Hz ending 1 s prior to salt injection.


(a)
+120 mM KCl
Control
(b)
+100 mM Na Benzoate
Control
(c)
+100 mM Na Benzoate
Control
+100 mM Na Benzoate
+0.5 μM NIG
(d)
+120 mM KCl
+1 μM VAL
Control
+120 mM KCl
+1 μM VAL
+0.5 μM NIG
Delayed Light Emission, Arbitrary Units
Time, ms

ated; 0.5 μM nigericin (nigericin; chlorophyll = 1:10) eliminated this KCl induced enhancement. Thus, it is clear that H^+ gradient is necessary for seeing the effect of ψ_l on millisecond delayed light. One micromolar valinomycin, present in all cases, did not significantly effect the proton gradient build up during preillumination.

No enhancement of 6 to 100 μs delayed light emission intensity was observed in response to a KCl-jump either in dark adapted or preilluminated chloroplasts (Fig. 31). Sodium benzoate injection also caused no enhancement of the microsecond delayed light emission (data not shown). Thus, it is clear that activation energy for microsecond delayed light is not provided by membrane potential.

4. Estimate of the Light Generated Membrane Potential after a Single Flash

An earlier attempt [98] to calibrate the light generated membrane potential, ψ_l, by comparing the enhancement of delayed light emission by ψ_l to that caused by salt-jump membrane potentials was done under multiple illumination conditions and, thus, is not suitable for comparison with estimates from the 515 nm absorption changes determined for single turnover flashes. The lack of salt-jump enhancement of delayed light emission after a single flash (Fig. 30) would appear to prevent the use of this technique for calibrating ψ_l after a single flash. However, a simultaneous injection of sodium benzoate with KCl allows a KCl induced enhancement of millisecond delayed light emission to be observed after a single flash (Fig. 32 (a)). Using previous estimates [98] for potassium and chloride permeabilities, a 60 mM KCl (final concentration) injection should induce a maximum potential of 67.1 mV. According to Crofts et al. [92], $\frac{L'}{L} = \exp(F\psi_s/RT)$, where L'

Fig. 31. Delayed light in the 6 to 100 μs range following a salt(KCl)-jump.

Logarithmic plot of delayed light emission decay in the 6 to 100 μs range. The chlorophyll concentration was 5 μg/ml and the suspension medium was 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH) with 1 μM valimonycin present. Samples were preilluminated for 30 s with laser pulses given at a rate of 32 Hz ending 1 s prior to salt injection. Decays are after a single flash given 0.5 s after an injection of KCl to a final concentration of 120 mM (■—■—■) or with no salt injection (o—o—o).

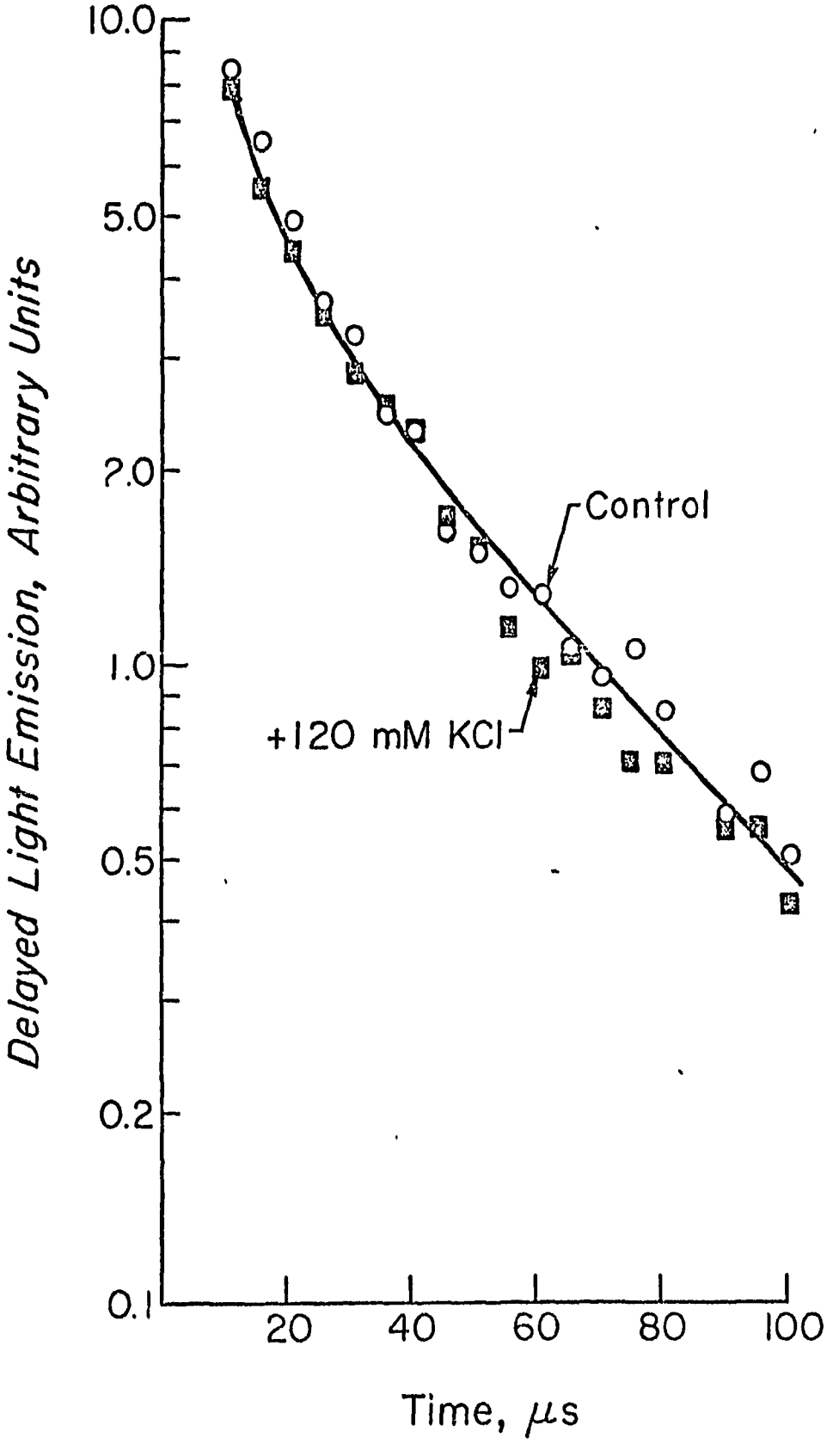
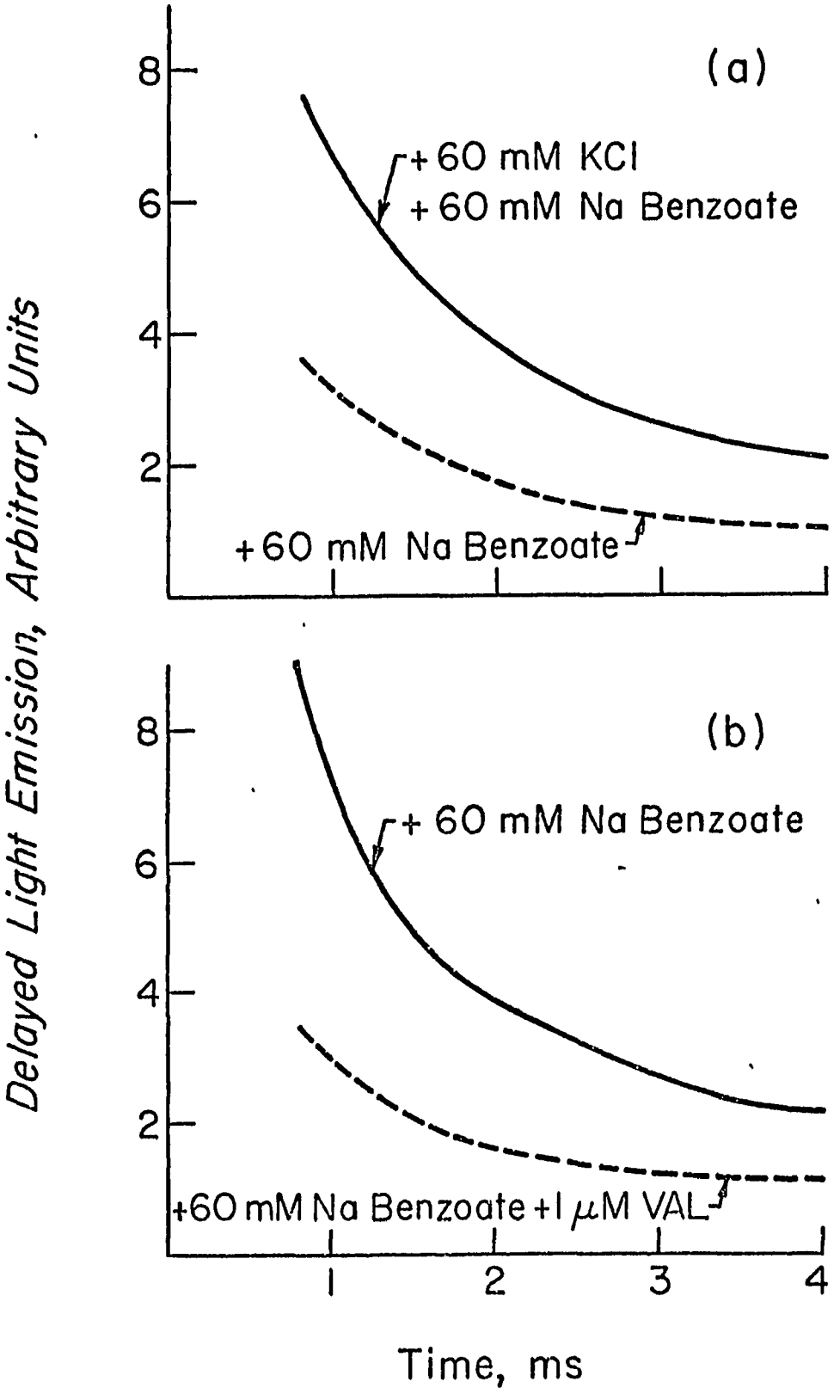

10.0
5.0
2.0
1.0
0.5
0.2
0.1
Delayed Light Emission, Arbitrary Units
Control
+120 mM KCl
20
40
60
80
100
Time, μs

Fig. 32. Delayed light in the 0.6 to 4 ms range after a single excitation flash following injection of sodium benzoate.

Delayed light emission decays in the millisecond range following injection of sodium benzoate. The chlorophyll concentration was 5 µg/ml and the suspending medium was 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH). All decays are after a single flash given 0.5 s prior to salt injection. (a) injection of sodium benzoate to a final concentration of 60 mM (- - -) or simultaneous injection of sodium benzoate and KCl to a final concentration of 60 mM each. 1 µM valinomycin was present. (b) injection of sodium benzoate to a final concentration of 60 mM with (- - -) and without (———) 1 µM valinomycin.


(a)
+60 mM KCl
+60 mM Na Benzoate
+60 mM Na Benzoate
(b)
+60 mM Na Benzoate
+60 mM Na Benzoate +1 μM VAL
8
6
4
2
1
2
3
4
Delayed Light Emission, Arbitrary Units
Time, ms

and L are the delayed light emission intensities with and without a salt-jump; ψ_s is the salt-jump potential; and R, F, and T are the gas constant, Faraday, and absolute temperature, respectively. For a 60 mM KCl salt-jump $L'/L = \exp(67.1 \text{ mV}/25.4 \text{ mV}) = 14$. However, the experimentally determined L'/L is only 2.14 (Fig. 32 (a)). This unexpectedly low value can be explained in two ways: (1) the membrane potential which effectively alters delayed light emission is smaller than the potential generated across the entire thylakoid membrane, and (2) only a certain fraction of the total delayed light emission is sensitive to membrane potential. These two possibilities are developed below.

(1) The idea that the total potential established across the thylakoid membrane is not effective in altering delayed light emission seems reasonable since the recombining charge pair, giving rise to delayed light emission, may not bridge the entire thickness of the thylakoid membrane. This was suggested by Evans and Crofts [99] for charge separation across the chromatophore membrane of Rhodopseudomonas capsulata (see Discussion). The effective membrane potential, ψ_D, will then be given by $\psi_m \times (1/d)$, where ψ_m is the total membrane potential across the thylakoid membrane, d is the thickness of the thylakoid membrane, and l is the distance, normal to the membrane surface, between the recombining charges. From Fig. 32 (a), $\exp(\psi_D/25.4 \text{ mV}) = 2.14$ giving $\psi_D = 19.3$ mV and $d/l = \psi_s/\psi_D = 67.1 \text{ mV}/19.3 \text{ mV} = 3.47$. From salt-jumps at various salt concentrations the average value of d/l was found to be 3.55 (Table 5). Since the thylakoid membrane thickness is estimated to be 40 Å [100], then l is ~11 Å. By measuring L'/L the membrane potential is calculated from $\psi_m = (3.55) \times (25.4 \text{ mV}) \times \ln(L'/L)$. In order to determine the value of ψ_l, the ratio of delayed light emission intensity after a single

TABLE 5

SALT-JUMP ENHANCEMENT OF MILLISECOND DELAYED LIGHT EMISSION

Data is from salt-jump enhancement of delayed light emission in the 1 to 4 ms range following a single saturating 10 ns flash given to dark-adapted Alaska pea chloroplasts at a chlorophyll concentration of 5 μg/ml with 1 μM valinomycin present. KCl and sodium benzoate were injected simultaneously 0.5 s after the excitation flash to give a final concentration of sodium benzoate of 60 mM and of KCl as indicated. The membrane potential generated by the salt-jump (ψ_s) and that indicated by delayed light emission (ψ_D) are calculated as in Results. The ratios of the delayed light amplitudes with and without salt-jump (L'/L) are obtained from decay data such as in Fig. 32 (a), d is the thylakoid membrane thickness, and l is the distance, normal to the membrane surface, between the recombining charges.

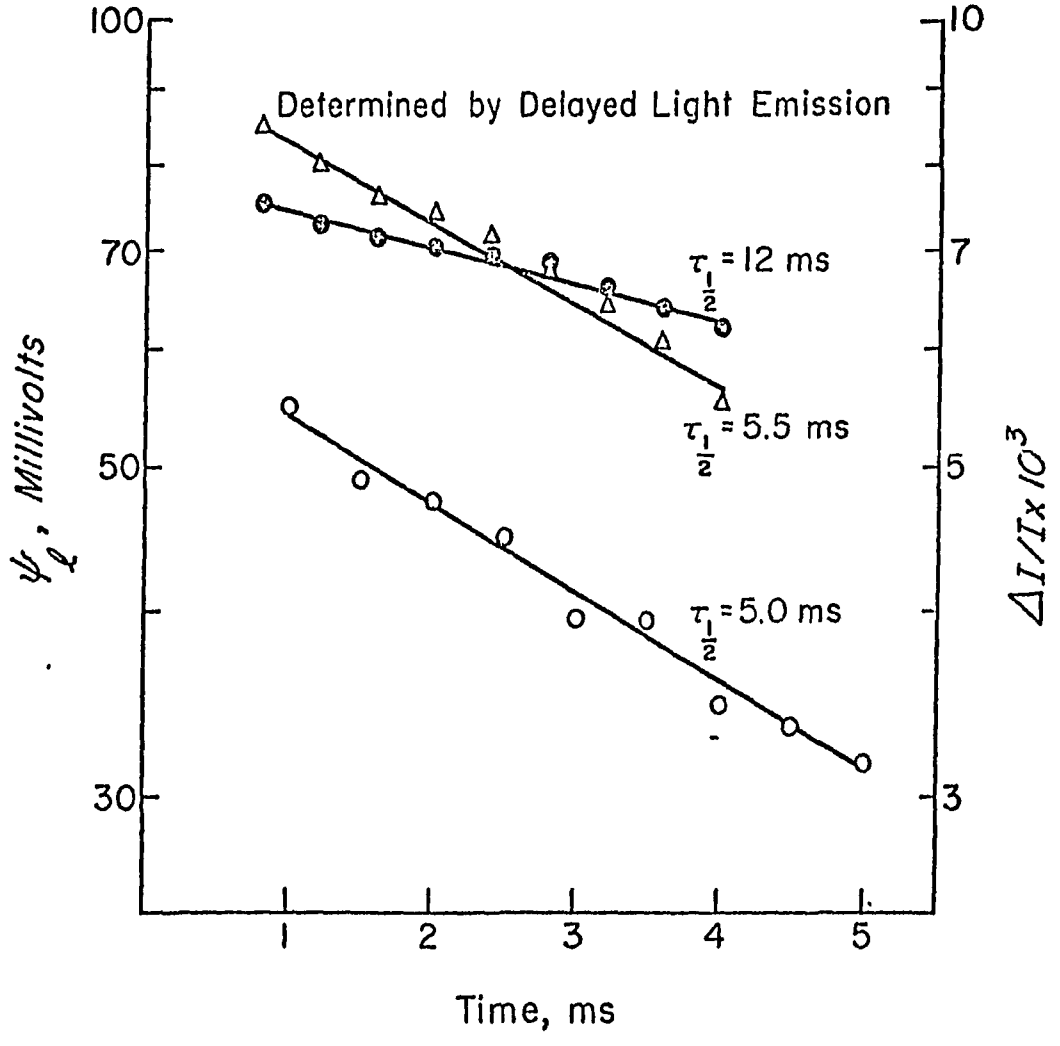
Final KCl Concentration (mM)	ψ_s, mV	L'/L	ψ_D, mV	$\psi_s/\psi_D = d/l$
120	73.3	2.28	20.9	3.50
60	67.1	2.14	19.3	3.47
60	67.1	2.09	18.7	3.58
60	67.1	2.07	18.5	3.63
15	47.7	1.70	13.5	3.54
4	25.9	1.33	7.24	3.57
				(avg. = 3.55)

flash in the presence and absence of 1 μM valinomycin was determined. As noted earlier, the effect of ψ_1 after a single flash was made observable by injection of sodium benzoate (Fig. 32 (b)). Using the formula given above, the decay in the millisecond range was calculated from Fig. 32 (b), and is plotted in Fig. 33 (Δ—Δ—Δ). For comparison, the decay of the 518 nm absorption change is also shown in Fig. 33 (o—o—o) and it had decay kinetics similar to those of ψ_1. From the decay of ψ_1, indicated by the 518 nm absorption change (Fig. 24), maximum value for ψ_1, calibrated using delayed light emission after a single flash and extrapolated to zero, is 128 ± 10 mV. The calculation of the rate of decay of ψ_1 assumes that the substrate for delayed light emission decays at the same rate with or without valinomycin. The close correspondence between the calculated ψ_1 decay and the observed 518 nm absorption change decay, therefore, suggests that the decay of $P^+_{680}Q^-$ is not strongly dependent on ψ_1. This conclusion was already made based on the lack of membrane potential effect on the microsecond delayed light emission, which is due to $P^+_{680}Q^-$ recombination.

(2) The second possibility is that only a portion of the delayed light emission intensity is sensitive to membrane potential. The Boltzmann factor, exp (67.1 mV/25.4 mV) = 14, would, therefore, not govern the ratio of total delayed light emission intensity (L'/L), but only a fraction of it. Then, $L = L_c + L\psi$ and for a 67.1 mV salt-jump generated membrane potential $L' = L_c + 14\ L\psi$, where L_c and $L\psi$ are portions of the delayed light emission intensity insensitive and sensitive to membrane potential, respectively. From Fig. 32 (a) L'/L = 2.4 and, thus, $L\psi = 0.096\ L_c$, i.e. only about 10% of the delayed light emission intensity L, seen in the absence of potential, is sensitive to membrane potential. The fraction

Fig. 33. Decay of the light generated membrane potential determined by delayed light and the 518 nm absorption change.

Logarithmic plot of the light generated membrane potential, ψ_1, as determined by salt-jump enhanced delayed light, after a single flash, calculated by two methods: (△—△—△), assuming that only a portion of ψ_1 is effective in altering the delayed light emission intensity and (●—●—●), assuming that only a portion of the total delayed light emission intensity is sensitive to ψ_1. (See text for details.) The logarithmic plot of the 518 nm absorption change ($\Delta I/I$), after a single flash, is also shown (o—o—o).


Determined by Delayed Light Emission
$\tau_{\frac{1}{2}}$ = 12 ms
$\tau_{\frac{1}{2}}$ = 5.5 ms
$\tau_{\frac{1}{2}}$ = 5.0 ms
ψ_ℓ, Millivolts
$\Delta I/I \times 10^3$
Time, ms
100
70
50
30
10
7
5
3
1
2
3
4
5

sensitive to the potential varies with the value of ψ_s used as the starting point for this calculation, thus, making this approach extremely suspect. Nevertheless, ψ_1 was calculated from the data of Fig. 32 (b) and plotted in Fig. 33 (●—●—●). The decay rate of ψ_1, calculated in this manner, is significantly slower (12 ms) than the decay rate as measured by 518 nm absorption change (5 ms). We consider this interpretation of the effect of ψ_1 on delayed light emission much less satisfactory than the former ((1), above).

Addition of ferricyanide was used to diminsh Photosystem I activity by chemically oxidizing P_{700}, the Photosystem I reaction center, in the dark. Complete blockage of Photosystem I activity has been reported [101] to decrease the 515 nm absorption change by 50%. In our chloroplasts, 5 mM ferricyanide (1000 ferricyanide:chlorophyll) reduced the 518 nm absorption change by 44% and ψ_1, calculated using the delayed light emission method described under (1) above, by 49%. As expected, lower concentrations of ferricyanide caused only partial blockage of Photosystem I activity and smaller decrase in membrane potential. For 0.5 mM ferricyanide the 518 nm absorption change was reduced by 28% and the membrane potential, calculated by delayed light emission, by 20%.

D. Discussion

1. The Need for a Proton Gradient

Our ability to measure thylakoid membrane potential effects on delayed light emission after a single turnover flash has provided new information unavailable from phosphoroscope measurements. For both light generated membrane potentials and KCl induced diffusion potentials, no effect on microsecond or millisecond delayed light emission is seen after a single

excitation flash (Figs. 25, 27 (a) and 30 (a)). With preillumination, however, enhancement of delayed light in the millisecond range by light generated membrane potentials and KCl salt-jump diffusion potentials are observable (Figs. 27 (b) and 30 (d)). The requirement for preillumination may arise from a dependence on the light generated proton gradient across the thylakoid membrane. This is shown by the elimination of the preillumination effect by nigericin (Fig. 27 (c)), similar decay kinetics of the preillumination enhancement of delayed light emission and the H^+-ion efflux measured with a pH electrode (Fig. 28) and the effect of benzoate (which produces a proton gradient, see the following section) in revealing the enhancement of delayed light emission after a single flash. As noted earlier, previous results on light generated membrane potential and salt-jump diffusion potential effect [85, 87, 88, 94, 98] were obtained with techniques that involved preillumination of the sample, thus, the requirement for preillumination and proton gradient was not appreciated. The requirement for a proton gradient suggests that millisecond delayed light emission may originate from the back reaction of Q^- with a protonated form (ZH^+) of Z [89].

2. The Effects of Sodium Benzoate

In previous salt-jump experiments, sodium benzoate was noted to cause a much greater stimulation of emission than other sodium salts [88, 89]. Crofts had suggested (see Kraan et al., [89]), on the basis of work on anion uptake in chloroplasts [102], that benzoate can penetrate the thylakoid membrane in the protonated form and thereby generate a pH-gradient. Such a behavior is well established for other anions in both chloroplasts [102] and mitochondria [103]. In our experiments: (i) salt jump enhancement

of delayed light emission can be observed after a single excitation flash if sodium benzoate is present (Fig. 30 (b)); (ii) injection of sodium benzoate allows the effects on delayed light emission of the light generated membrane potential to be seen after a single excitation flash (Fig. 32 (a) and (b)); and (iii) nigericin eliminates both these effects of sodium benzoate. These results are consistent with the interpretation that sodium benzoate establishes a proton gradient across the thylakoid membrane.

3. The Light Generated Thylakoid Membrane Potential after a Single Flash

The ability of sodium benzoate to establish a proton gradient across the thylakoid membrane was used to calibrate the magnitude of the light generated potential following a single excitation flash. In the presence of valinomycin, simultaneous injection of sodium benzoate and KCl followed by a single flash allowed the enhancement of delayed light emission by a known diffusion potential to be established (Fig. 32 (a)). The effect of the light generated membrane potential, after a single flash, was also observable following injection of sodium benzoate (Fig. 32 (b)) and was used to estimate a value for the initial light generated membrane potential of 128 ± 10 mV with decay kinetics in the millisecond range similar to those seen for 518 nm absorption changes (Fig. 33). The magnitude of 128 mV agrees well with recent estimates of 105-135 mV based on voltage dependent ionophores [104], but is higher than earlier estimates of 50 mV based on the 518 nm absorption change [95, 101] and 30-70 mV determined with microelectrodes placed across an intact chloroplast [105, 106].

Inhibition of Photosystem I activity by 5 mM ferricyanide caused a 50% reduction of both the 518 nm absorption change and the membrane potential

calculated by delayed light emission. The parallel decrease lends support to the belief that millisecond delayed light emission is influenced by the membrane potential.

The method of calculating the membrane potential from the millisecond delayed light emission suggests that the distance between the recombining charges is only about 11 Å perpendicular to the thylakoid membrane surface. This is in agreement with the conclusion [107] that the Photosystem II donor and acceptor are located within the lipophilic area of the membrane (also see section 5).

4. The Lack of a Membrane Potential Effect on Microsecond Delayed Light

There is no observable effect of membrane potential on delayed light emission in the microsecond range, except for the addition of 1 μM gramicidin (1 gramicidin;5 chlorophyll) (Fig. 26). However, at this high concentration inhibition may be a consequence of secondary effects of gramicidin since no inhibition of delayed light emission occurs with 0.1 μM gramicidin (Fig. 26), a concentration which completely eliminates 518 nm absorption changes in our samples. One possible explanation for the general lack of sensitivity in the microsecond range is that the charge separation is over only a small transverse membrane distance, for example ≤ 5Å, in which case a 70 mV salt-jump potential would cause only a 20% increase, which would be barely discernable above the microsecond delayed light emission signal noise level.

The lack of effect of membrane potential on microsecond delayed light emission leads to the conclusion that in the microsecond range the thylakoid membrane potential does not provide the activation energy for delayed light

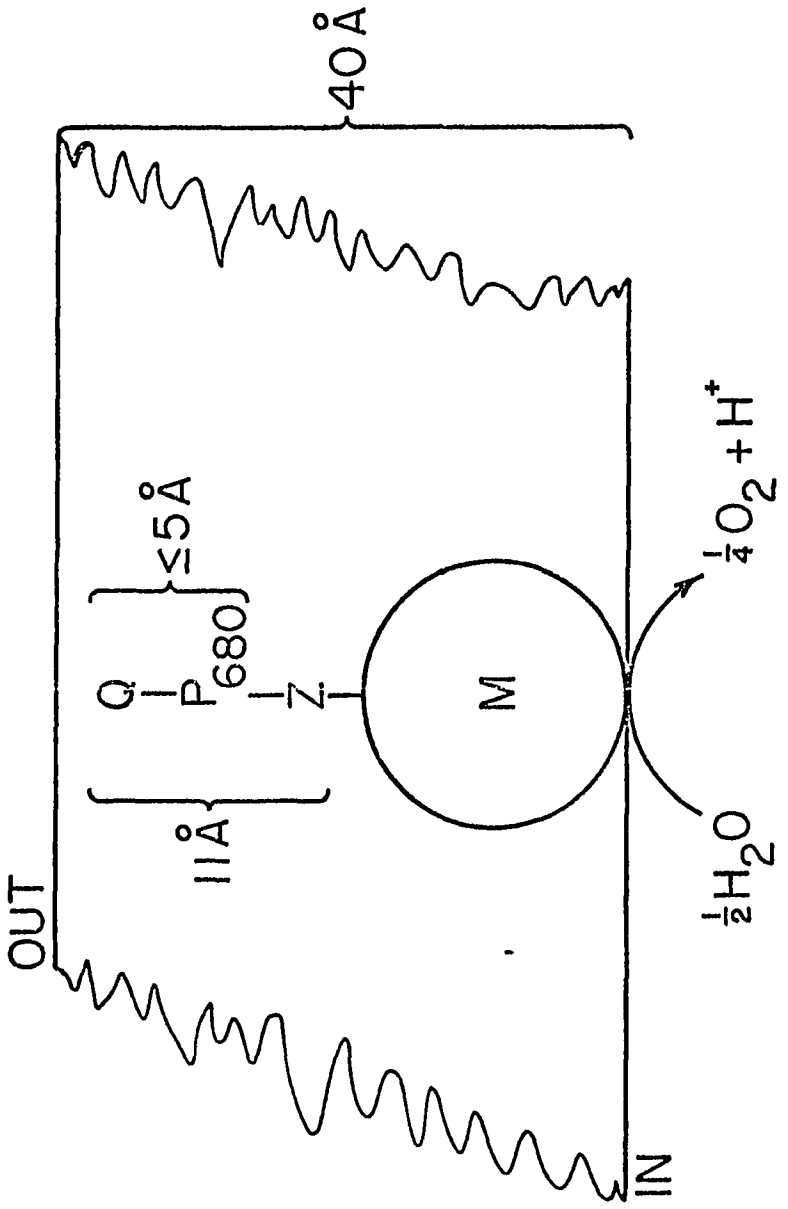
emission. In the millisecond range, however, we do observe enhancement of delayed light by membrane potential, but, only if a proton gradient is present and this is apparently consistent with the concept of a lowering of the delayed light emission activation energy [92].

5. A Possible Membrane Model for Photosystem II

Fig. 34 shows a working model for Photosystem II components in the thylakoid membrane that is consistent with the data presented in this chapter. The small initial charge separation and incomplete spanning of the membrane by a single step, suggested here, is similar to current views of the reaction center of photosynthetic bacteria [108, 109] and is consistent with recent observations on the 515 nm absorption change also indicating only partial spanning of the membrane by the $P^{+}_{680}Q^{-}$ couple [P. Joliot, personal communication to Dr. Govindjee]. The placement of Q on the outer side of the membrane is based on the proposal and the experiments with silicomolybdate of Zilinskas [110]. The placement of p_{680} closer to the outside of the membrane was suggested by Arntzen *et al.* [111] on the basis of their iodination experiments (also see [107]). Finally, the location of the oxygen evolving system (M) on the inner side of the membrane is suggested by proton release experiments of Fowler and Kok [112].

Fig. 34. Proposed model for the arrangement of Photosystem II components in the thylakoid membrane.

A model for the possible arrangement of Photosystem II components in the thylakoid membrane based partly on interpretations of data in this work (also see Trebst, [107]). P_{680} is the reaction center chlorophyll and the primary electron donor, Q is the "primary" electron acceptor, Z is the first secondary electron donor, and M is the chemical species which accumulates four positive charge equivalents before reacting with water to evolve oxygen.


40Å
≤5Å
11Å
Q
P_{680}
Z
M
OUT
IN
$\frac{1}{4}O_2 + H^+$
$\frac{1}{2}H_2O$

CHAPTER VI

DELAYED LIGHT EMISSION DECAY IN THE 6 TO 340 μS RANGE AFTER A SINGLE FLASH: TEMPERATURE EFFECTS

A. Introduction

The sensitivity of delayed light emission (microsecond to second range) to temperature has been demonstrated by several methods. Light emission can be obtained from preilluminated samples by slow heating (the glow curve technique) [30, 113] with four or more burst of emission at different temperatures [36, 83, 114, 115]. Rapid heating (temperature-jumps) also causes preilluminated chloroplasts to emit a pulse of delayed light emission [116, 117, 118]. The amplitude and decay kinetics of delayed light emission are temperature dependent in the time range of seconds [16, 82, 117, 119, 120, 121], milliseconds [82, 120], and microseconds [59].

A detailed temperature study was carried out on the decay kinetics of delayed light emission in the second region from the green alga Chlorella in the 0 to 45 °C range [117]. An Arrhenius plot of the delayed light emission decay rate constant for Chlorella had a discontinuity between 10 and 20 °C, consisting of a segment of almost zero slope. Up to this time a nonlinear Arrhenius plot for delayed light emission decay has not been reported for any other materia. Other processes in plants vary in a discontinuous manner with temperature and in many cases this is correlated with changes in membrane lipid fluidity [122]. Succinate oxidation activity in chill-insensitive plants (potato tubers, cauliflower buds, and beet root) varies in a monotonic fashion while in chill-sensitive plants (tomato fruit, sweet potato and cucumber fruit) in a discontinuous manner with

temperature [123, 124]. Measurements on electron spin resonance (ESR) of fatty acid probes, incorporated in membranes, indicated discontinuous membrane lipid fluidity changes in the chill-sensitive but not in the chill-insensitive plants. Arrhenius plots of $NADP^+$ photoreduction were linear for chill-insensitive plants (lettuce and pea) but had a single discontinuity for chill-sensitive plants (tomato and bean) [125]. The effect of temperature on the electron transport reaction and on ESR spin labels in the blue-green alga Anacystis showed discontinuities in both the parameters at the same temperature [126]. Hydrogen ion movement across the thylakoid membrane of spinach is also temperature sensitive [127, 128] having a discontinuity at 18 ^oC, which is the same temperature ESR probes [129] indicated a membrane phase change.

In the present chapter, we report the temperature characteristics of delayed light emission decay, after a single 10 ns laser pulse, in the 6 to 340 μs range for chloroplasts from both chill-sensitive (beans) and chill insensitive (pea, lettuce, and spinach) plants. Also, the temperature dependence of thylakoid membrane lipid fluidity as determined by ESR spectra of fatty acid spin labels and differential scanning calorimetry (DSC) is presented. Supporting data on delipidated membranes and extracted lipids are also included.

The current theory for the origin of delayed light emission is the recombination of P^+_{680} and Q^-, where P^+_{680} is the oxidized from of the reaction center chlorophyll a and Q^- is the reduced form of the primary acceptor [17]. This theory predicts a linear dependence of delayed light emission on light intensity. Jones [29], Stacy et al. [32], and Lavorel [130] reported I^2 dependence for millisecond delayed light emission in samples given low in-

tensity illumination of pulse lengths 1.7 ms, 3.5 ms, and 0.28 ms, respectively. Zankel [59] observed a linear dependence on intensity of microsecond delayed light emission following a microsecond flash. We show in this chapter that microsecond delayed light following a 10 ns flash is linearly dependent on light intensity at intensities an order of magnitude lower than used by Zankel [59].

B. Materials and Methods

Chloroplasts were extracted from leaves of pea, beans, lettuce, and spinach according to procedures described in Chapter II. The lettuce and spinach were purchased at a local market, and the peas and beans were grown in the laboratory. Intact cells of the blue-green alga Anacystis nidulans grown at 22 ^{o}C were also used, and references for growth media and procedures are cited in Chapter II.

Extraction of lipids was carried out using the modified Bleigh-Dyer method [131] and lipid-water dispersions were made as follows: (1) the lipids were extracted in chloroform which was blown off with nitrogen, (2) water was added to lipids in the ratio of 3:1 (w:w), and (3) mixing was done for 5 min at 50 ^{o}C with a vortex mixer and forced movement through an 18 gauge needle with a syringe. This procedure is similar to that of Bangham et al. [132], which produces multilamellar liposomes.

Microsecond and millisecond range delayed light emission decays after a single flash were measured using apparatus described in Chapter II. Chlorophyll a fluorescence measurements were made by replacing the nitrogen laser with light from an incandescent lamp, passed through a water filter and a Corning CS 4-96 glass filter. Sample temperature was regulated

for both measurements by using a quartz water jacketed fluorimeter cuvette (Precision Cell model 54FL).

ESR spectra of the fatty acid spin label 12 nitroxide stearate (12NS) obtained from Syva, Palo Alto, California, were measured in a Varian (Model E-9) ESR spectrometer with a Varian (Model V-4540) variable temperature controller. Samples were held in glass tubes 9.3 cm long having a 2 mm inside diameter, and the temperature was measured inside the tube with a thermistor (Fenwal Electronics, Type GB32J2). Spectra were recorded within 60 to 90 min after introduction of the spin label to obtain the largest signals. By 120 min after addition of the spin label, the signal amplitude had decreased by one-half due to chemical reduction of the paramagnetic nitroxide group by membrane components [133]. For all ESR data, spectra were taken at a particular temperature after the sample had been at that temperature for 5 min. The following ESR spectrometer settings were used: the scan time was 4 min, the time constant 1 s, microwave power 20 mW, and modulation amplitude 1 gauss. As a control to check the ESR method, 12NS was incorporated into a lipid-water dispersion of 1-2-dipalmitoyl-L-lecithin (lecithin/water, 34/66, w/w) and a characteristic [134] sharp phase transition was observed at 41 °C.

The method used to incorporate 12NS into chloroplasts or lipid-water dispersions was as follows: (1) 12NS in ethanol was added to a small test tube, and the ethanol was blown off with nitrogen, (2) chloroplasts at a chlorophyll concentration of 15 mg/ml or lipid and water were added to give a final molar ratio of chlorophyll to 12NS of 15 to 1, and (3) thorough mixing was accomplished by using a vortex mixer for 5 min at 50 °C.

Differential scanning calorimeter measurements were made using a Perkin-Elmer model DCS-2 calorimeter with a refrigeration unit. The temperature and enthalpy scales were calibrated with known masses of pure indium and lead. Samples were placed in hermetically sealed 20 μl aluminum pans and the reference pan contained the phosphate buffer described earlier. As a control to check the operation of the calorimeter, scans were made on a lipid-water dispersion of 1-2-dipalmitoyl-L-lecithin (lecithin/water, 34/66, w/w) and a characteristic [135] sharp peak was observed at 41 ^{o}C and a small pretransition at 34 ^{o}C.

The fluorescence of tryptophan was measured in a Perkin-Elmer spectrofluorometer (model MPF-3) fitted with a temperature regulation unit. These measurements were carried out on chloroplasts delipidated according to previously described procedures [136, 137].

Glutaraldehyde fixed spinach chloroplasts were prepared according to a procedure described by Zilinskas and Govindjee [138]. Absorption measurements, used to determine the extent of glutaraldehyde fixation were made with a Cary-14 spectrophotometer. Photochemical activity of fixed chloroplasts was determined by the rate of oxygen evolution, with ferricyanide as an electron acceptor, under saturating continuous illumination, with a Yellow Springs Instrument platinum/Ag-$AgCl_2$ Clark electrode and Model 53 oxygen monitoring system.

C. Results

1. Microsecond Delayed Light Emission Dependence on Actinic Light Intensity

In order to better understand the possible mechanisms that might lead to the generation of delayed light emission, light curves were made

at very low light intensities (where multiple hits of the reaction center would not occur) to test whether delayed light emission intensity follows I or I^2 dependence. The samples were kept in complete darkness for 15 min to allow for the decay of any energy trapping states. A log-log plot (Fig. 35) of microsecond delayed light intensity shows a slope of unity, indicating that a one quantum process is involved. This finding is in agreement with results reported for samples given microsecond flash illumination [59] but does not agree with a slope of two found for millisecond delayed light emission [29, 32, 130].

2. Temperature Dependence of Delayed Light Emission Decay and Chlorophyll a Fluorescence Yield

The decay of delayed light emission in the 6 to 340 μs range in Alaska pea chloroplasts at 3 or 25 °C is shown in Fig. 36 (a) and (b). 0.1 μM DCMU (DCMU:chlorophyll = 1:50) was present in these samples to avoid any complications that may be caused by temperature induced changes in the electron flow rate from Photosystem II to the intersystem electron carrier pool. The rapidly decaying components at $t < 100$ μs show no change in kinetics with temperature, but the delayed light emission intensity decreases with decreasing temperature. This lowering of intensity is actually due to decreases in the amplitudes of slower decaying components. A temperature effect on the decay kinetics is observed beyond 120 μs after the flash, with the exponential decay lifetimes being 58 μs at 25 °C and 100 μs at 3 °C. This agrees well with temperature effects on delayed light emission decay kinetics in the 65 to 250 μs range in spinach chloroplasts [59]. Samples without DCMU present had the same temperature effects as seen in Fig. 36 (not shown).

Fig. 35. Delayed light emission intensity at 60 μs versus the incident flash intensity at low light levels.

A logarithmic plot of delayed light emission at 60 μs after a 10 ns excitation flash versus the logarithm of the incident flash intensity. A single excitation flash was given after a dark adaptation period of 15 min. Chloroplasts were at a chlorophyll concentration of 5 μg/ml and the lowest incident laser light intensity shown (5×10^{11} quanta/cm^2) is equivalent to 1 quanta absorbed per 2×10^4 chlorophyll molecules.

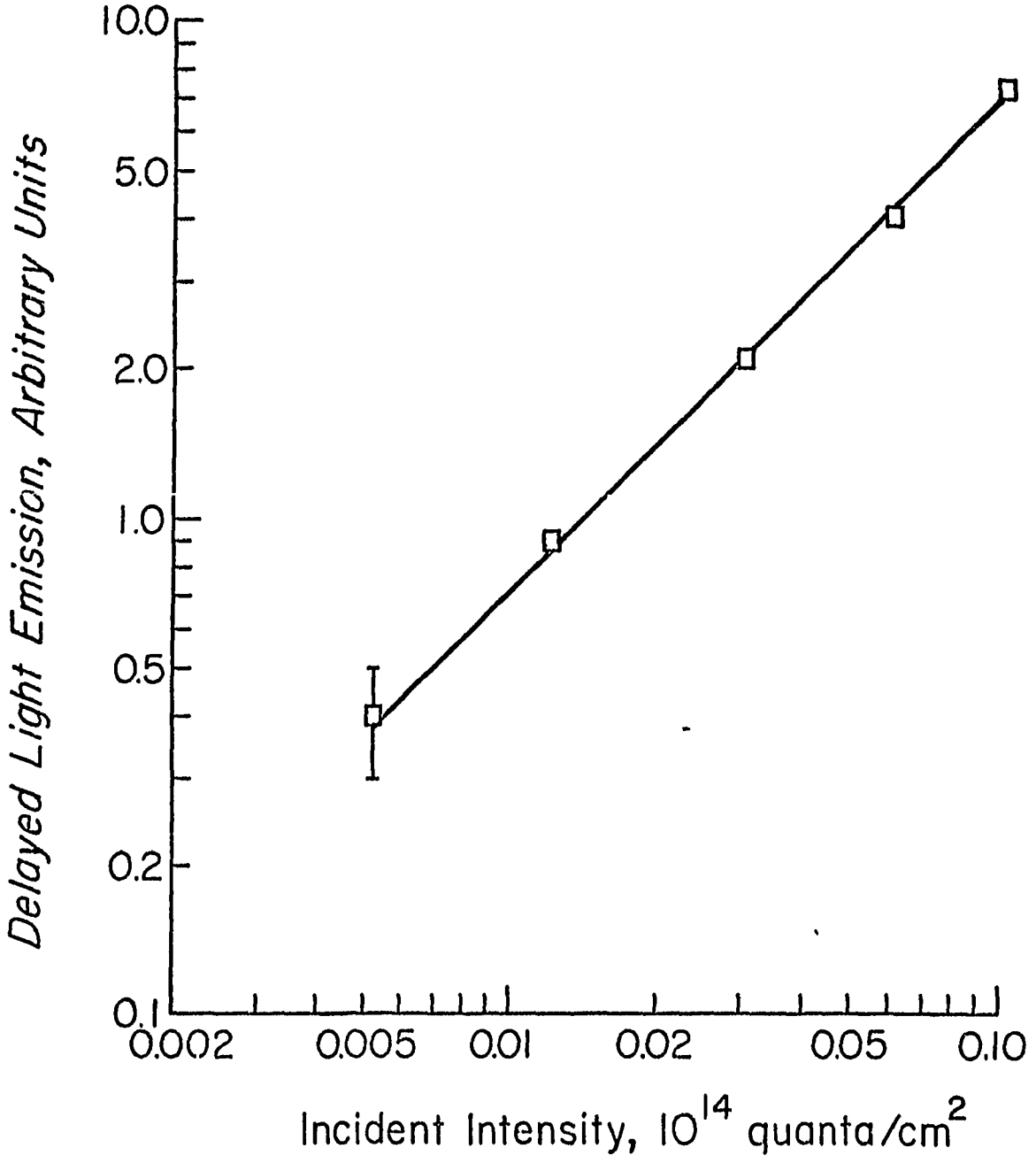
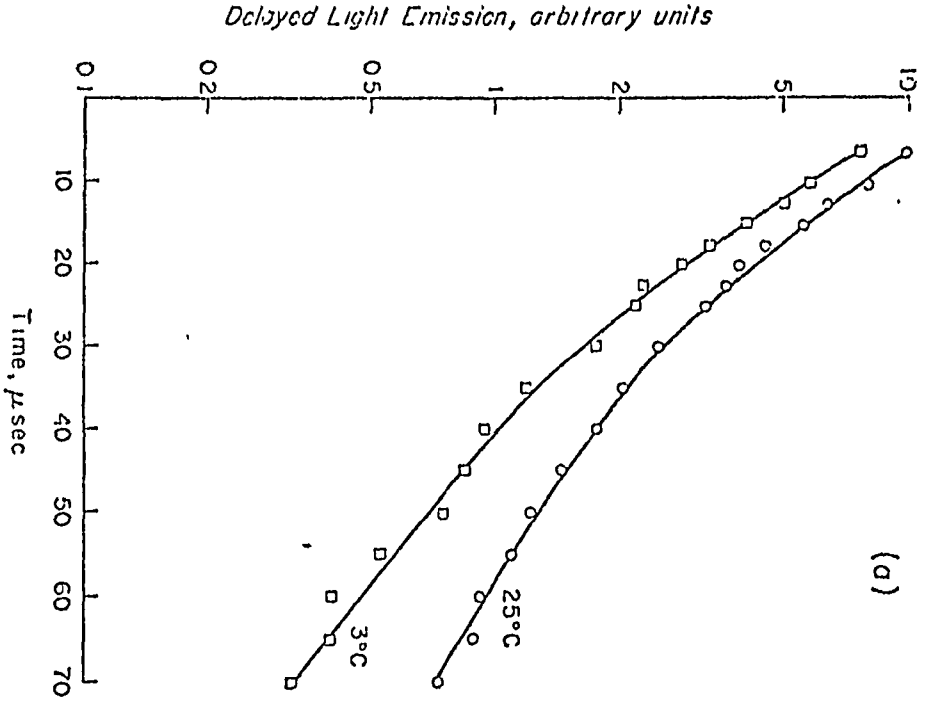
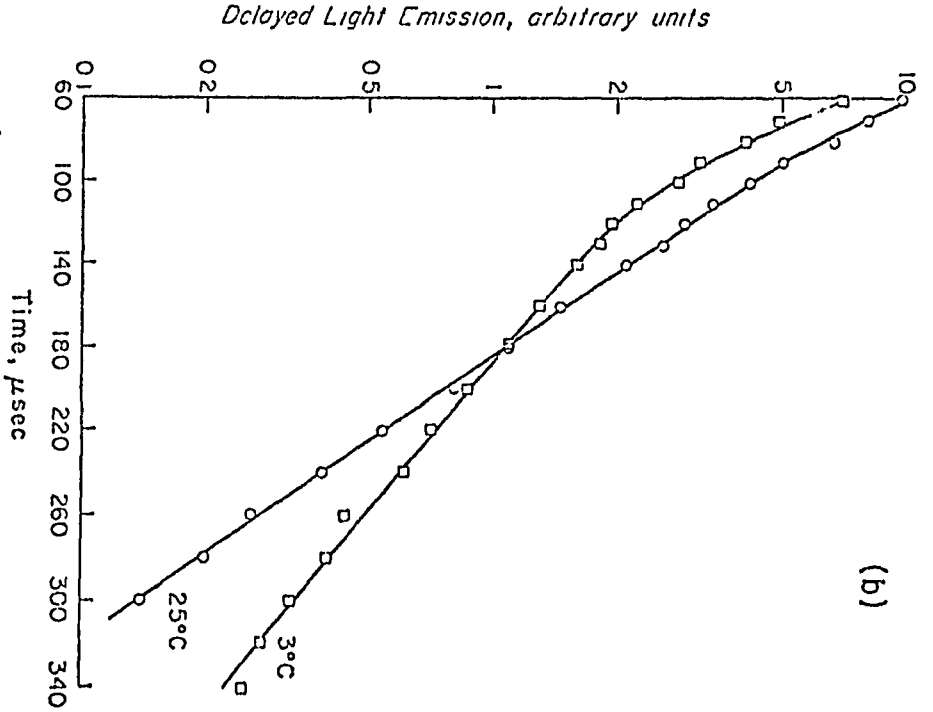

10.0
5.0
2.0
1.0
0.5
0.2
0.1
Delayed Light Emission, Arbitrary Units
0.002
0.005
0.01
0.02
0.05
0.10
Incident Intensity, 10^{14} quanta/cm^2

Fig. 36. Delayed light emission decay in the 6 to 340 μs range at 3 and 25 °C.

Logarithmic plot of the delayed light emission decay after a final flash in a series of eight or more flashes given at a rate of 1 flash/2s. Alaska pea chloroplasts at a chlorophyll concentration of 5 μg/ml with 0.1 μM DCMU present. Decays are shown for 3 and 25 °C in the 6 to 70 μs range (a), and the 60 to 340 μs range (b).


Delayed Light Emission, arbitrary units
0.1
0.2
0.5
1
2
5
10
10
20
30
40
50
60
70
Time, μsec
3°C
25°C
(a)


Delayed Light Emission, arbitrary units
0.1
0.2
0.5
1
2
5
10
60
100
140
180
220
260
300
340
Time, μsec
25°C
3°C
(b)

The rise in chlorophyll a fluorescence yield after an excitation flash, for chloroplasts at 3 °C is the same as at 25 °C (Fig. 37).

Arrhenius plots of the exponential decay constant k for 120 to 340 μs delayed light showed a linear curve for lettuce but a discontinuity between 12 and 17 °C for bush bean chloroplasts (Fig. 38).

Upon illumination a light generated membrane potential, ψ_1, of 50 to 135 mV is established across the thylakoid membrane [95, 104, see Chapter V]. It has been suggested [93] that the activation energy for delayed light emission, E_a, will be decreased by ψ_1 as follows: $E_a' = (E_a - \psi_1)$. We looked for this effect in our Arrhenius plots in samples that have, ψ_1, eliminated in the microsecond range by gramicidin D (see Chapter V) (Fig. 39 (a) and (b)), but found that the activation energies differ by only 15 mev, not 100 mev, the approximate value of ψ_1 in this time range (see Chapter V).

We then tested to see if discontinuities seen in Arrhenius plots might be due to large structural change in the thylakoid membrane. Glutaraldehyde fixation of chloroplasts was used to block large structural changes [139]. Glutaraldhyde fixed chloroplasts, used in our experiments were completely fixed according to previously established criteria [138], and retained 30% of their capacity to evolve oxygen. Arrhenius plots of the delayed light emission decay constant in both fixed and unfixed spinach chloroplasts showed discontinuities (Fig. 40).

3. Fatty Acid Spin Labelling of Thylakoid Membrane and Water Dispersions of Lipids

Fatty acid spin labels, such as 12NS, are stable paramagnetic molecules which may be incorporated into biological membranes and their

Fig. 37. Rise in chlorophyll *a* fluorescence yield in the 3 to 35 μs range at 3 and 25 °C.

A plot of the rise in chlorophyll *a* fluorescence yield, ϕ_f, following a final flash in a series of eight or more flashes given at a rate of 1 flash/2s. ϕ_0 is the level of fluorescence prior to an actinic flash. Alaska pea chloroplasts were at a chlorophyll concentration of 5 μg/ml; fluorescence data is shown at 25 °C (o—o—o) and 3 °C (□—□—□).

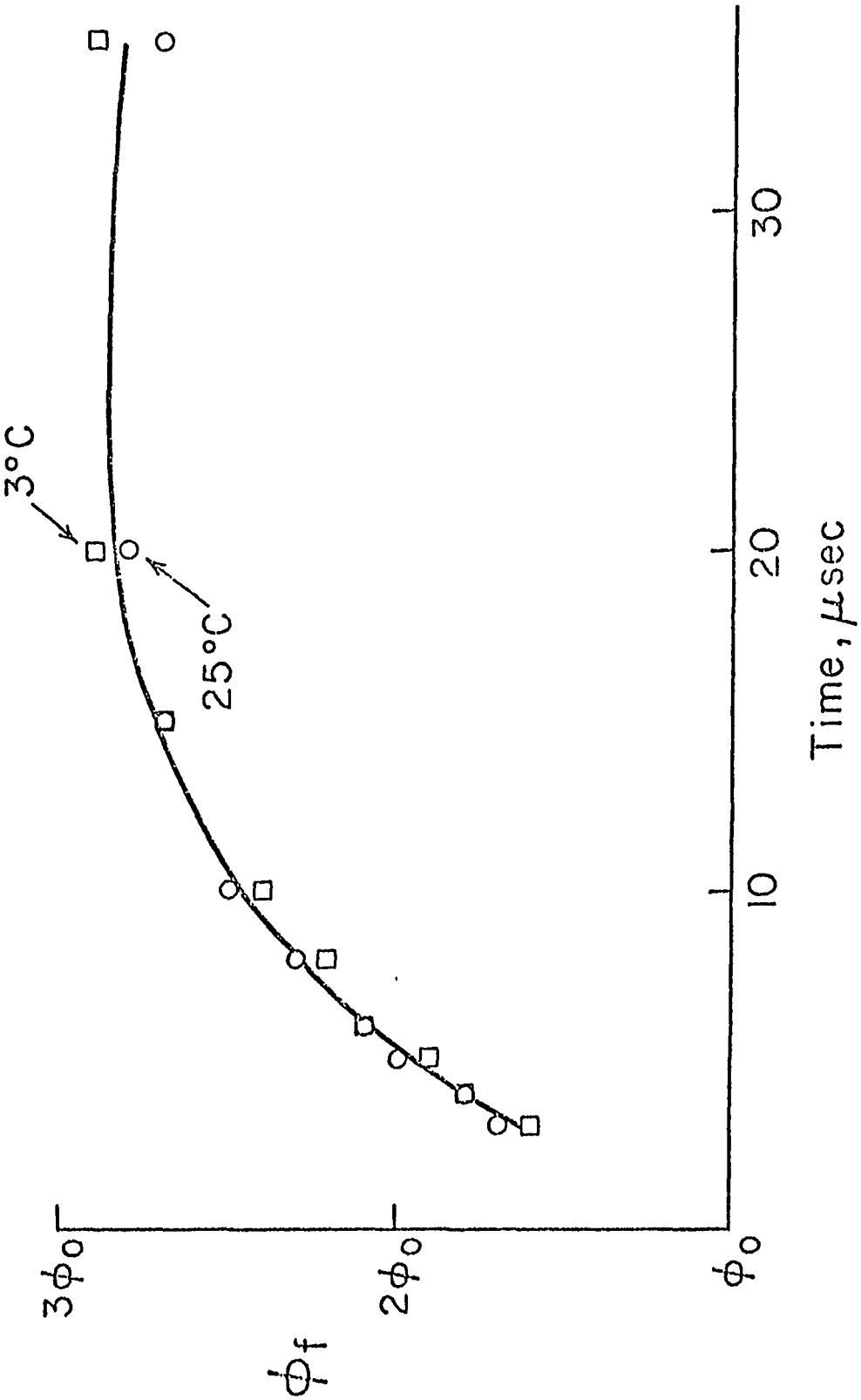

3°C
25°C
0
10
20
30
Time, μsec
3φo
2φo
φo
φf

Fig. 38. Arrhenius plot of the delayed light emission decay constant in bush bean and lettuce chloroplasts.

Arrhenius plots of the exponential decay constant, k, obtained from semilogarithmic plots of delayed light emission decay in the 120 to 340 μs range in bush bean chloroplasts (a) and lettuce chloroplasts (b). Both samples had 0.1 μM DCMU present and were at a chlorophyll concentration of 5 μg/ml. The activation energy, E_a, calculated from the Arrhenius plot slope is given in millielectron volts (mev).

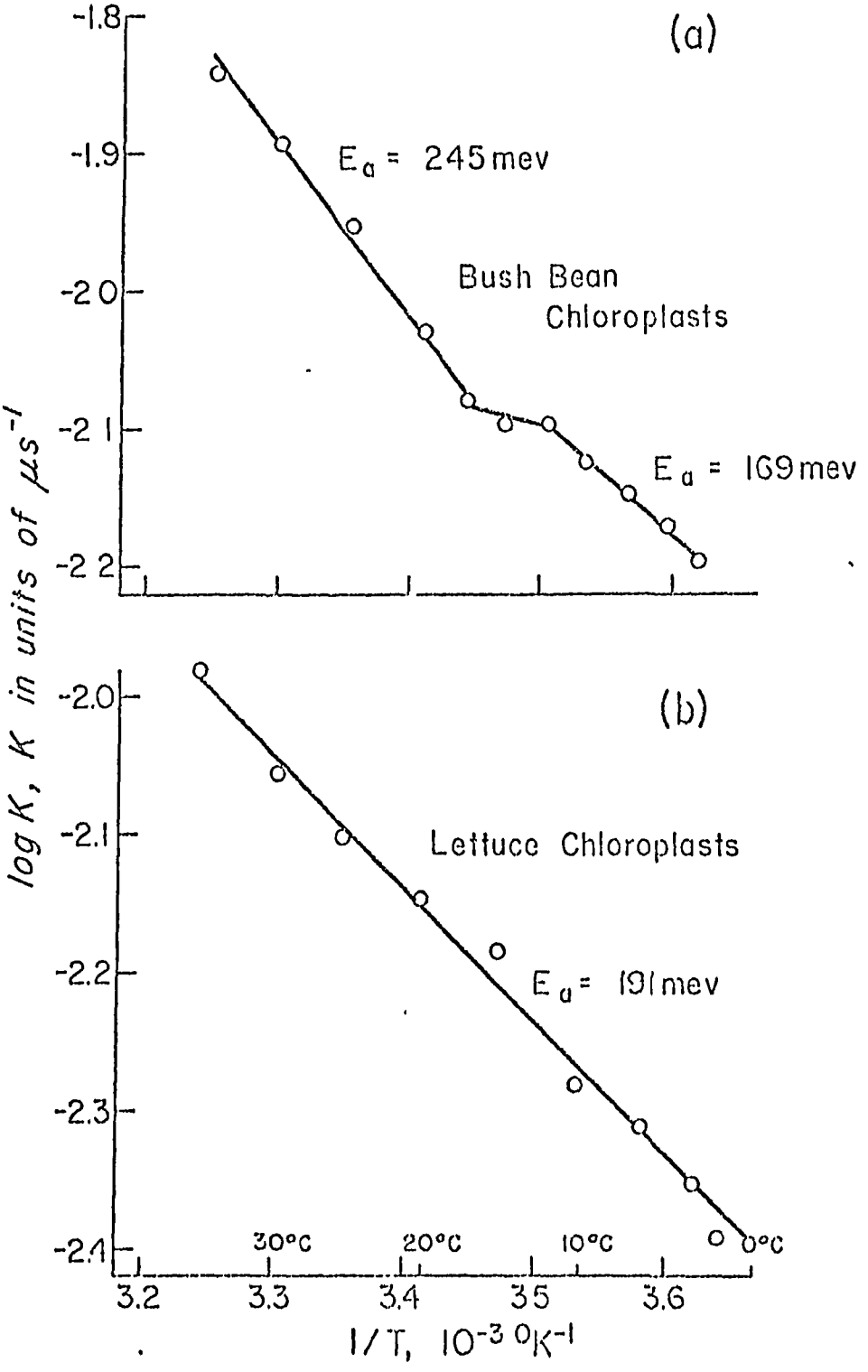

(a)
E_a = 245 mev
Bush Bean
Chloroplasts
E_a = 169 mev
(b)
Lettuce Chloroplasts
E_a = 191 mev
30°C
20°C
10°C
0°C
log K, K in units of μs^{-1}
1/T, 10^{-3} $^0K^{-1}$

Fig. 39. Arrhenius plot of the delayed light emission decay constant in Alaska pea chloroplasts with and without 1 μM gramicidin D.

Arrhenius plots of the exponential decay constant, k, obtained from semilogarithmic plots of delayed light emission decay in the 120 to 340 μs range in Alaska pea chloroplasts (a) and Alaska pea chloroplasts with 1 μM gramicidin D present (b). Chloroplasts were at a chlorophyll concentration of 5 μg/ml. The activation energy, E_a, calculated from the Arrhenius plot slope is given in millielectron volts (mev).

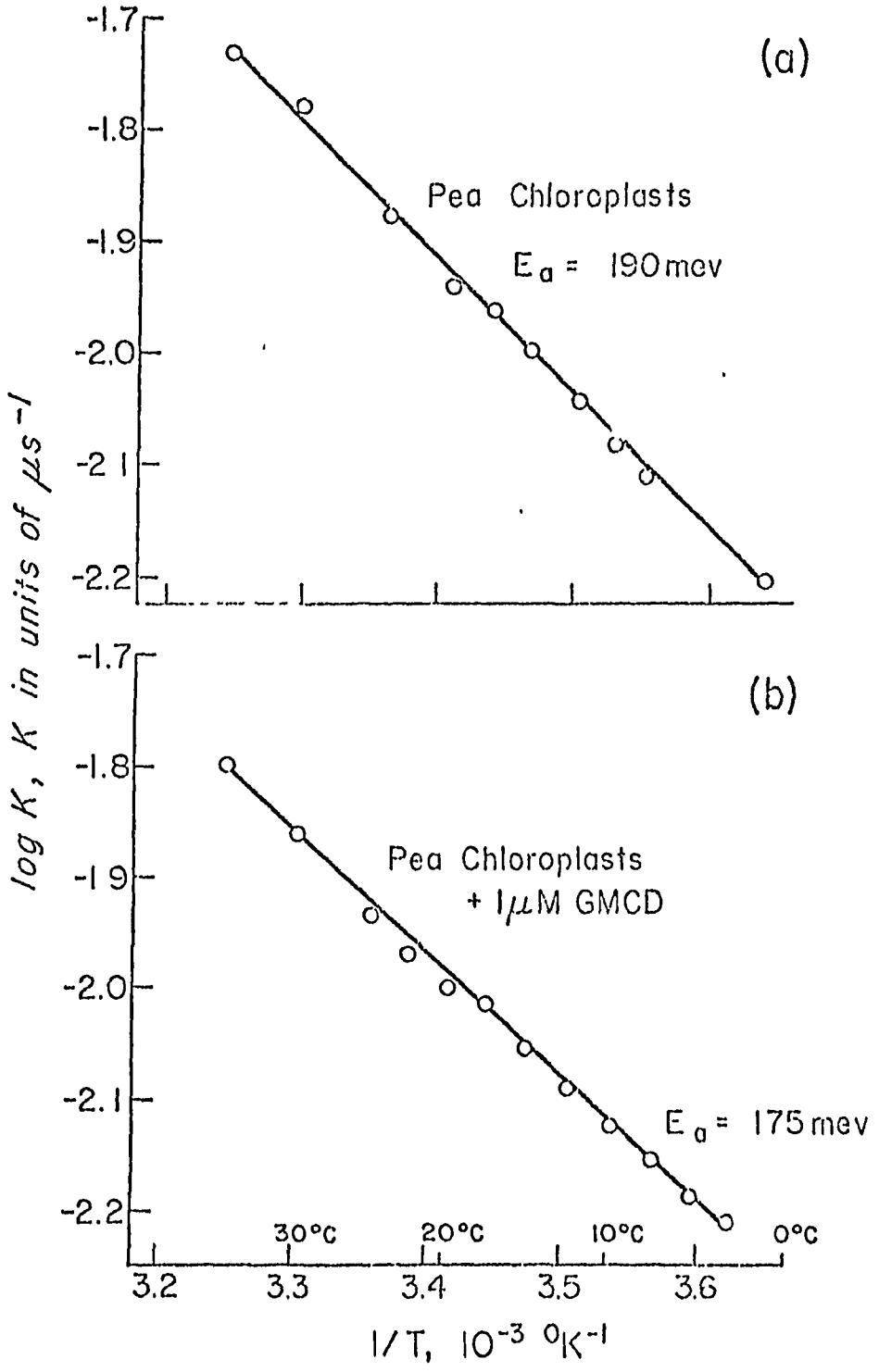
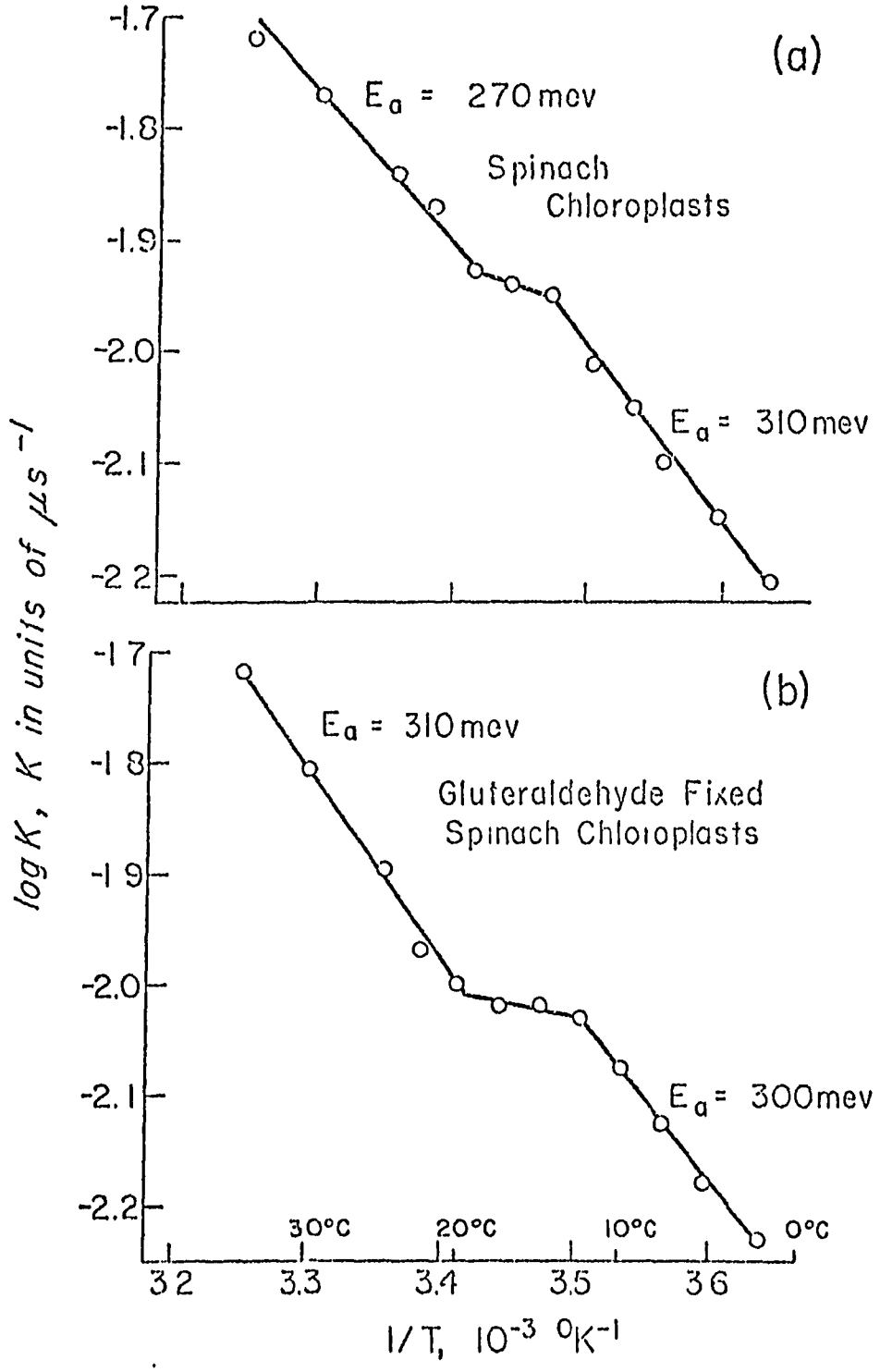

(a)
Pea Chloroplasts
E_a = 190 mev
(b)
Pea Chloroplasts
+ 1μM GMCD
E_a = 175 mev
log K, K in units of μs⁻¹
-1.7
-1.8
-1.9
-2.0
-2.1
-2.2
30°C
20°C
10°C
0°C
3.2
3.3
3.4
3.5
3.6
1/T, 10^{-3} °K⁻¹

Fig. 40. Arrhenius plot of the delayed light emission decay constant in unfixed and glutaraldehyde fixed spinach chloroplasts.

Arrhenius plots of the exponential decay constant, k, obtained from semilogarithmic plots of delayed light emission decay in the 120 to 340 μs range in unfixed (a) and glutaraldehyde fixed spinach chloroplasts (b). Chloroplasts were at a chlorophyll concentration of 5 μg/ml with 0.1 μM DCMU present. The activation energy, E_a, calculated from the Arrhenius plot slope is given in millielectron volts (mev).


(a)
E_a = 270 mev
Spinach
Chloroplasts
E_a = 310 mev
(b)
E_a = 310 mev
Gluteraldehyde Fixed
Spinach Chloroplasts
E_a = 300 mev
30°C
20°C
10°C
0°C
log K, K in units of μs^{-1}
1/T, 10^{-3} $^0K^{-1}$

magnetic resonance spectra provide information about the fluidity of the spin label's environment [140]. Various spin labels have been used to detect membrane lipid phase transitions in microorganisms [141, 142, 143], animal cells [123, 144, 145, 146] and plant cells [125, 126]. We used this technique to determine if the thylakoid membrane lipids in our samples might undergo phase transitions that would correlate with the delayed light emission Arrhenius plot discontinuities.

The hyperfine splitting constant ($2T_{11}$) in ESR spectra can be taken as an indicator of mobility of the spin label in membrane lipids [142]. Fig. 41 is a plot of $2T_{11}$ versus temperature for 12NS in bush bean chloroplast membranes and in a water dispersion of extracted lipids from the same chloroplasts. A change in the slope occurs between 10-20 ^{o}C for both preparations and can be interpreted as a change in the membrane lipid fluidity [142].

For membranes with a low viscosity, the hyperfine splitting peaks in ESR spectra are not resolved; instead, the rotational correlation time, τ_c, may be calculated from line broadening in the spectra [125]. The low viscosity condition existed for 12NS incorporated into Alaska pea chloroplast membranes and water dispersions of extracted lipids from the same chloroplasts. A plot of the rotational correlation time versus the reciprocal of temperature is linear (Fig. 42) and may be interpreted to indicate that no discontinuous change in membrane lipid fluidity occurs in Alaska pea chloroplasts in this temperature range [125].

4. Differential Scanning Calorimetry of Water Dispersions of Extracted Lipids

Differential scanning calorimetry has been used to indicate lipid

Fig. 41. ESR spectra hyperfine splitting constant versus temperature from 12NS in bush bean chloroplasts and a water dispersion of extracted bush bean lipids.

The maximum hyperfine splitting of ESR spectrum, $2T_{11}$, of 12NS versus the reciprocal of absolute temperature. The spin label was present at a concentration of one 12NS per 15 chlorophyll in (a) bush bean chloroplasts and (b) a lipid water dispersion (20 mg chlorophyll:300 mg water) of lipids extracted from bush bean chloroplasts.

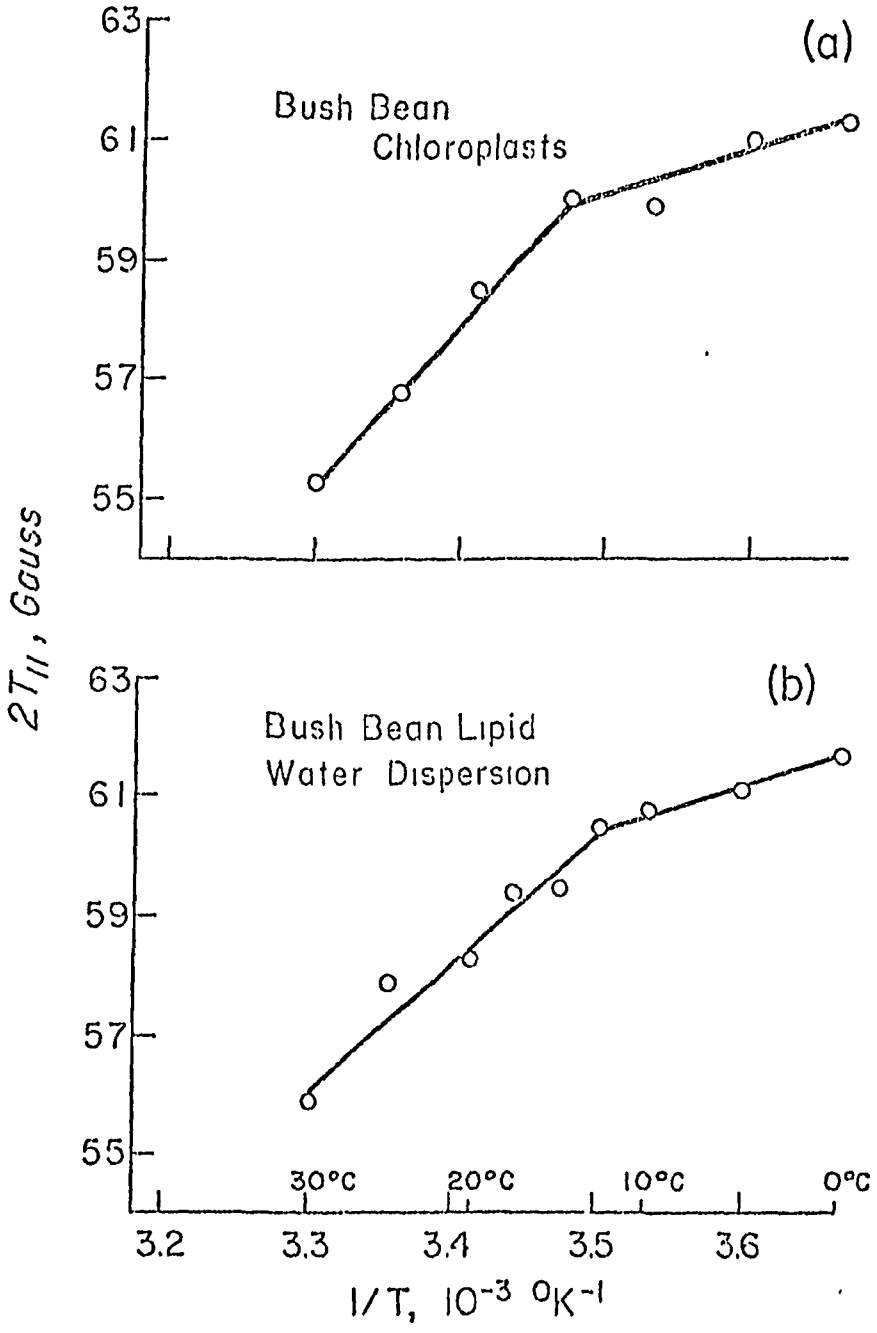
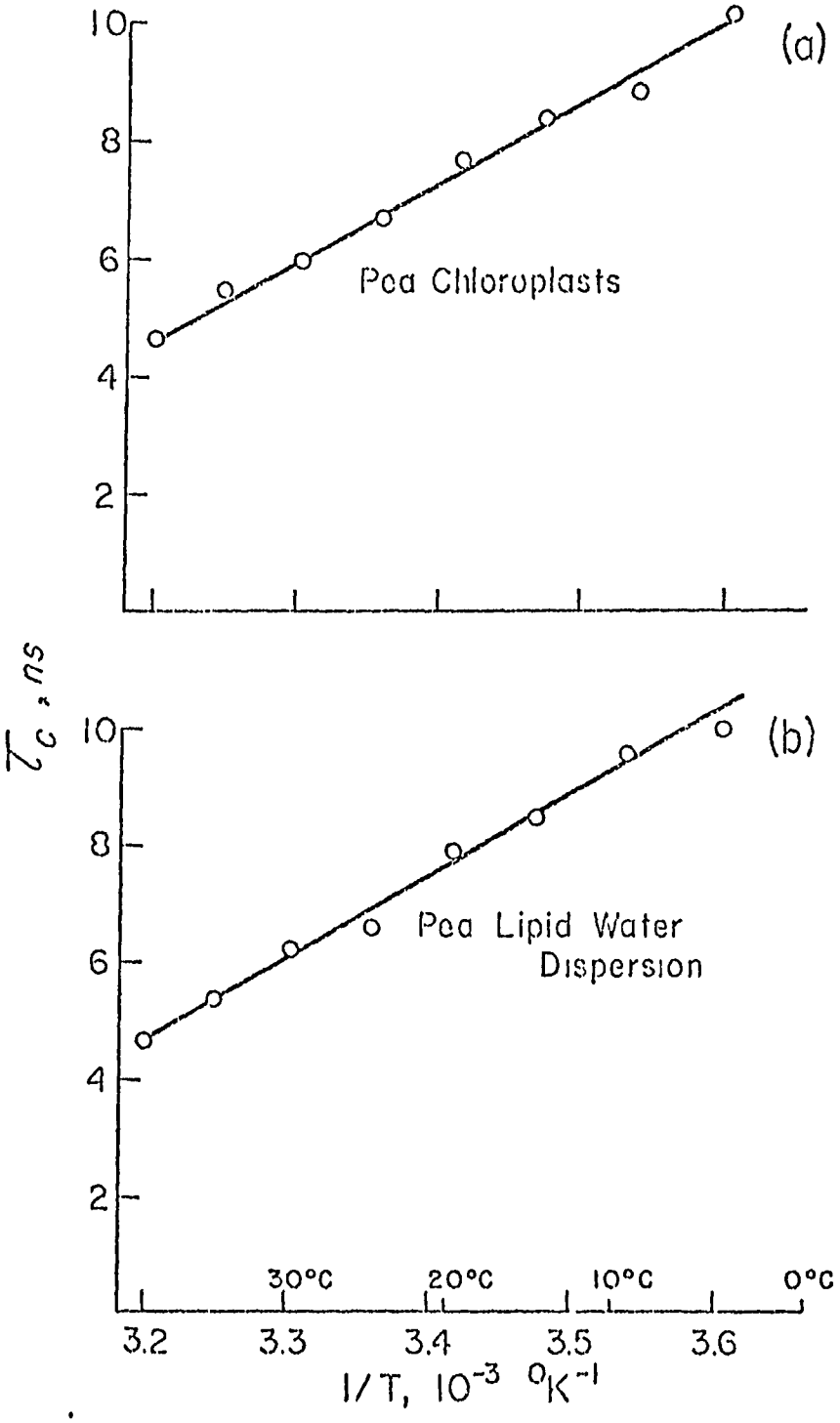

(a)
Bush Bean
Chloroplasts
(b)
Bush Bean Lipid
Water Dispersion
63
61
59
57
55
$2T_{II}$, Gauss
30°C
20°C
10°C
0°C
3.2
3.3
3.4
3.5
3.6
1/T, 10^{-3} $^{o}K^{-1}$

Fig. 42. ESR spectra rotational correlation time versus temperature from 12NS in Alaska pea chloroplasts and a water dispersion of extracted Alaska pea lipids.

The rotational correlation time, τ_c, of 12NS versus the reciprocal of absolute temperature. The spin label was present at a concentration of one 12NS per 15 chlorophyll in (a) Alaska pea chloroplasts and (b) a lipid water dispersion (20 mg chlorophyll:300 mg water) of lipids extracted from Alaska pea chloroplasts.


10
8
6
4
2
(a)
Pea Chloroplasts
τ_c, ns
10
8
6
4
2
(b)
Pea Lipid Water
Dispersion
30°C
20°C
10°C
0°C
3.2
3.3
3.4
3.5
3.6
1/T, 10^{-3} $^{o}K^{-1}$

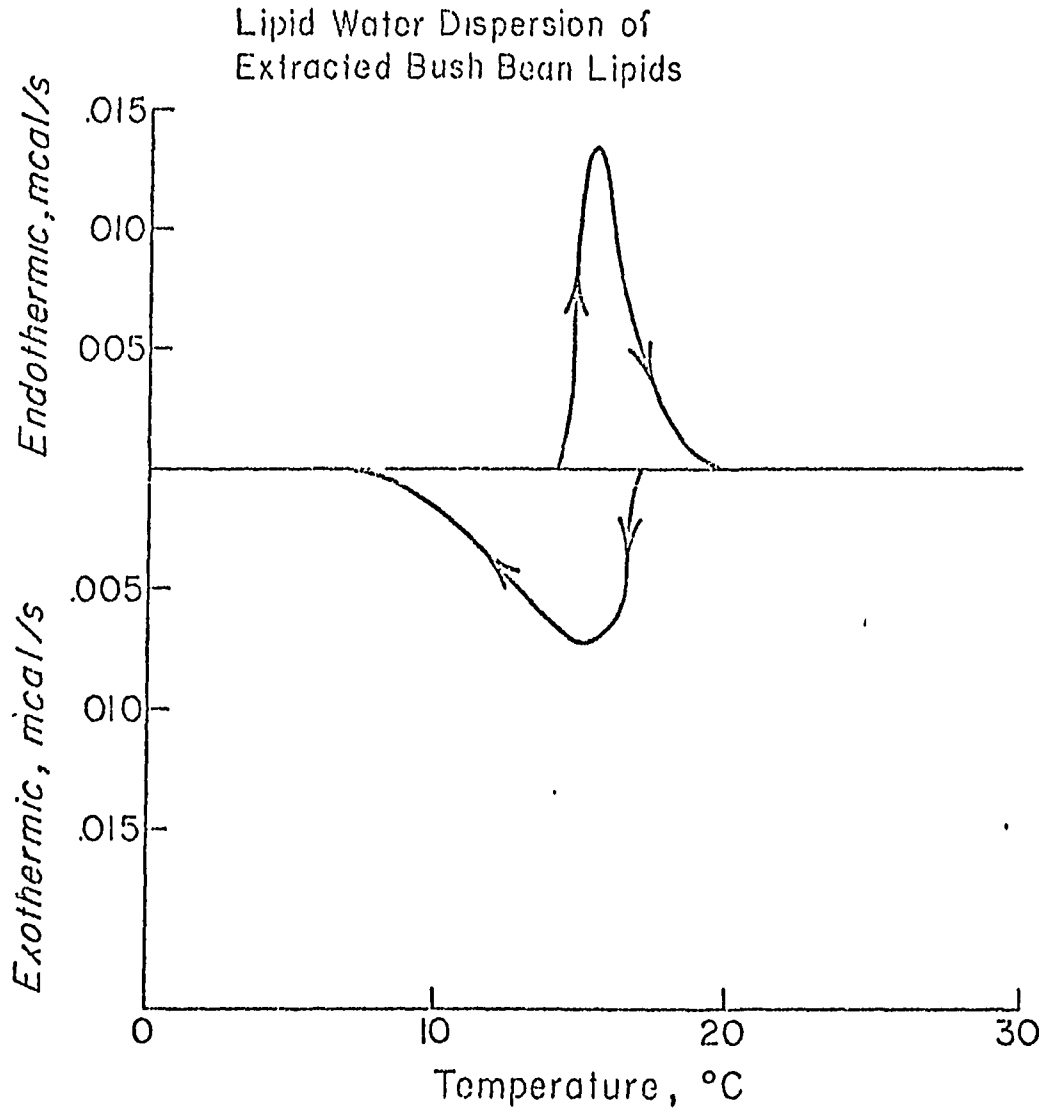
phase transitions in lipid-water dispersions, membrane fragments, and whole cells [135, 147-150]. Due to the low lipid mass of bush bean and Alaska pea chloroplasts, we were unable to observe transition peaks in whole membranes. (Earlier reports of transition peaks in whole membrane [147-150] were only made possible by the use of specially prepared sample pans having a seven fold greater volume than those available for our use.) However, by extracting chloroplast lipids and making water dispersions, approximately five times as much lipid mass could be placed in the sample pan than was possible with whole chloroplast membranes. Fig. 43 shows calorimetry scans for a water dispersion of lipids extracted from bush bean chloroplasts. Increasing and decreasing temperature scans are shown with the latter having the broadest transition (10 ^{o}C); both transition peaks occur at about 15 ^{o}C. Water dispersions of extracted lipids of Alaska pea chloroplasts did not show transition peaks, as expected.

5. Chlorophyll a Fluorescence as a Function of Temperature

ESR fatty acid spin labels have been used to demonstrate that membrane lipids of the blue-green alga Anacystis nidulans undergo a phase transition at approximately 13 or 24 ^{o}C in organisms grown at 28 or 38 ^{o}C respectively [126]. The chlorophyll a fluorescence from intact cells of Anaystis with DCMU present had a temperature dependence with a peak at the transition temperature [126, 151]. Measurements were made of chlorophyll a fluorescence in chloroplasts of Alaska pea, bush bean and spinach to see if any correlation might occur between the fluorescence temperature dependence and the delayed light emission, ESR and scanning calorimetry data. The chlorophyll a flurescence temperature dependence of Anacystis grown at

Fig. 43. Differential calorimeter scans of water dispersions of lipids extracted from bush bean chloroplasts.

Differential scanning calorimeter peaks for a water dispersion of lipids extracted from bush bean chloroplasts (lipid/water, 1/2.6, w/w). Samples of 3.6 mg total lipid mass were used, and the scanning rate was 5 ^{o}C/min.


Lipid Water Dispersion of
Extracted Bush Bean Lipids
Endothermic, mcal/s
.015
010
005
Exothermic, mcal/s
.005
010
.015
0
10
20
30
Temperature, °C

22 °C is observed to become maximal at 9 °C (Fig. 44), which agrees well with the earlier report [151]. However, no peak in the fluorescence temperature dependence is observed for Alaska pea, bush bean or spinanch chloroplasts (Fig. 44). Note that bush bean and spinach chloroplasts showed discontinuities in dealyed light emission decay rates, but Alaska pea chloroplasts did not. It seems that chlorophyll a fluorescence changes cannot be taken as a reliable indicator of membrane fluidity changes because bush bean chloroplasts and their lipids showed a negative correlation between fluorescence changes and the fluidity measured by ESR and calorimetry methods.

6. Tryptophyl Fluorescence as a Function of Temperature

The ESR and scanning calorimetry data provide information about the temperature effects on lipids especially when lipid-water dispersions are involved. To determine if membrane proteins might show discontinuous temperature behavior, the protein fluorescence of delipidated chloroplasts was measured at various temperatures. The fluorescence peaked at 330 nm (uncorrected spectra) when 280 nm excitation was used. This is the fluorescence peak from chloroplast protein tryptophyl residues [137]; it showed a linearly decreasing intensity with increasing temperature (Fig. 45) for both bush bean and Alaska pea delipidated chloroplasts.

D. Discussion

1. Microsecond Delayed Light Emission Is a One Quantum Process

The dependence of microsecond delayed light emission at low light intensities on the first power of the light intensity (I) (Fig. 35) confirms the observation of Lavorel [17] that I^2 dependence becomes an I dependence when the illumination time drops below 100 μs. The I dependence indicates

Fig. 44. Temperature dependence of chlorophyll a fluorescence intensity in Anacystis and chloroplasts of Alaska pea, bush bean and spinach.

Temperature dependence of chlorophyll a fluorescence intensity in Anacystis nidulans grown at 22 °C and chloroplasts of Alaska pea, bush bean and spinach. All samples had 1 μM DCMU present. Fluorescence measurements were made with the following chlorophyll concentrations: Anacystis, 1 μg/ml and all chloroplasts at 5 μg/ml. Fluorescence was excited by light from an incandescent lamp passed through a water filter and a Corning CS 4-96 glass filter and measured through a 686 nm interference filter. The intensity of the exciting light was 1.5 mW/cm^2 at the sample.

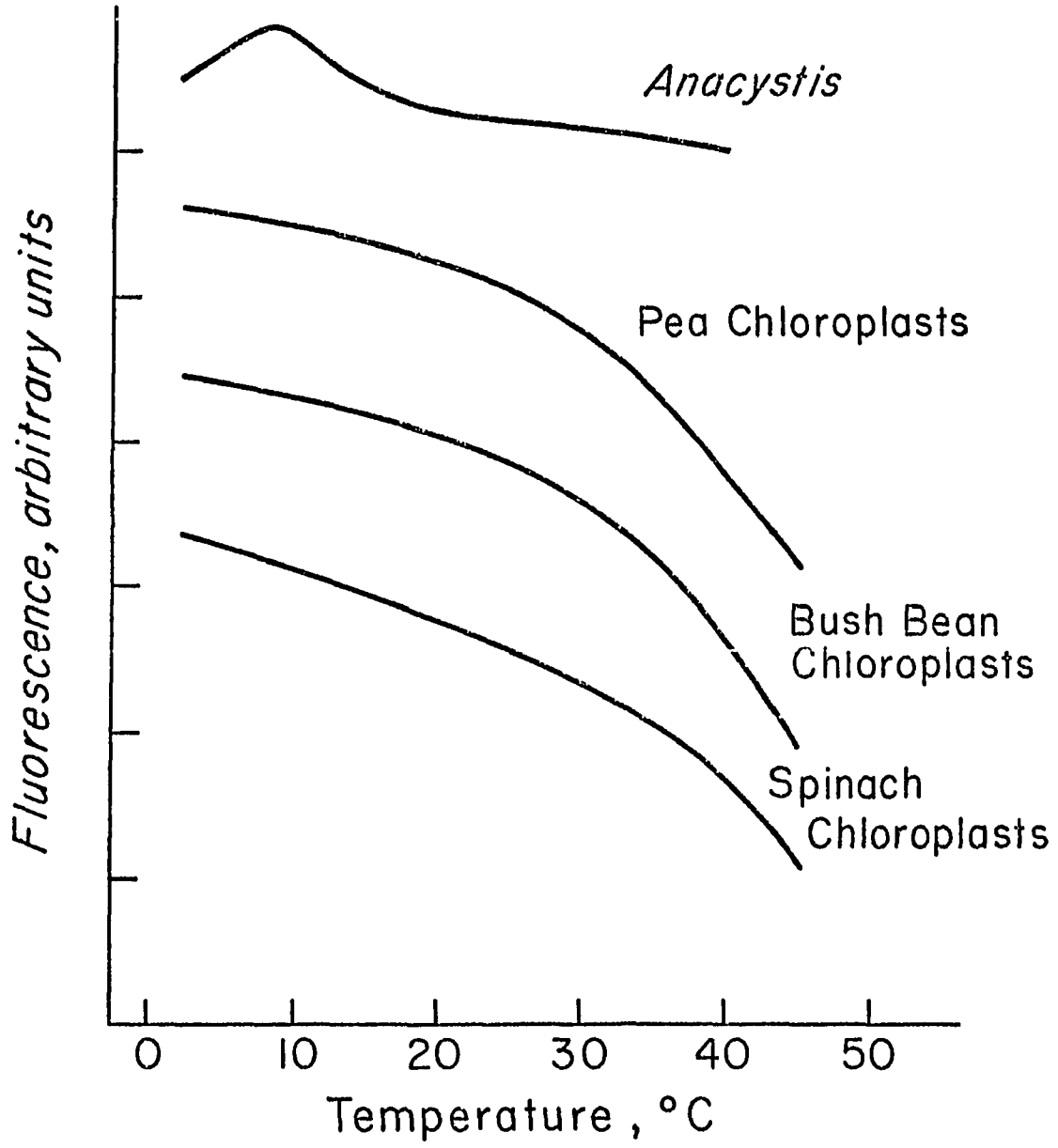
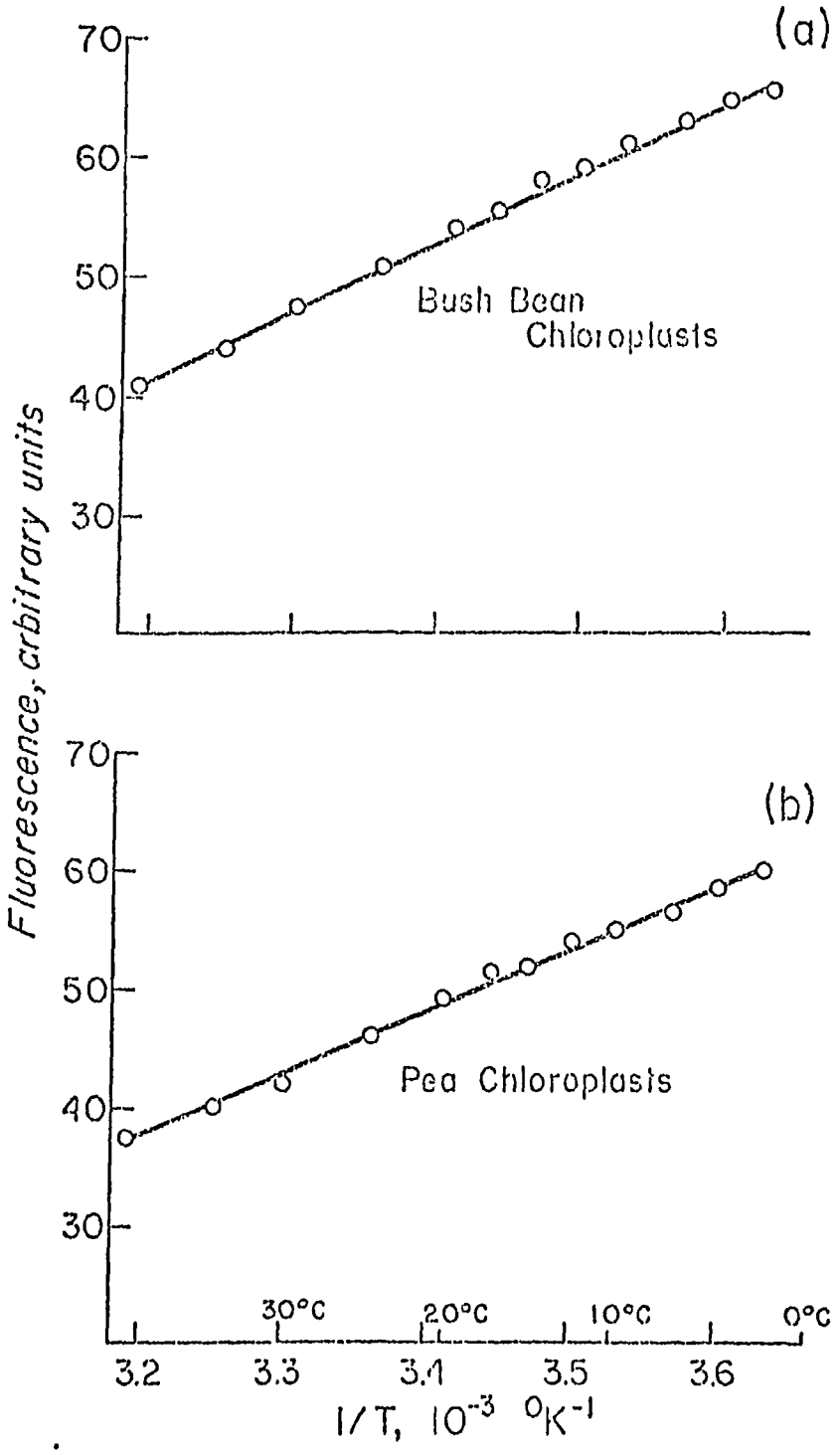

Anacystis
Pea Chloroplasts
Bush Bean Chloroplasts
Spinach Chloroplasts
Fluorescence, arbitrary units
0
10
20
30
40
50
Temperature , °C

Fig. 45. Temperature dependence of tryptophan fluorescence from delipidated bush bean and Alaska pea chloroplasts.

Temperature dependence of tryptophan fluorescence from delipidated bush bean chloroplasts (a) and Alaska pea chloroplasts (b). Fluorescence measurements were made with a Perkin-Elmer MPF-3 spectrofluorometer with a temperature regulated cuvette. Excitation was at 280 nm and fluorescence was observed at 330 nm with 3 nm bandwidth.


(a)
70
60
50
40
30
Bush Bean
Chloroplasts
(b)
70
60
50
40
30
Pea Chloroplasts
Fluorescence, arbitrary units
30°C
20°C
10°C
0°C
3.2
3.3
3.1
3.5
3.6
1/T, 10⁻³ °K⁻¹

that microsecond delayed light emission is a one quantum process. This suggests that theories requiring two quantum, such as electron-hole recombination [30, 31] and the triplet-triplet fusion [32], are not valid in the microsecond time range. The I dependence is consistent with the charge recombination theory of delayed light emission, since only one photon need be absorbed to generate the charge couple that can recombine to give delayed light emission.

2. Temperature Sensitivity of Microsecond Delayed Light Emission

The kinetics of delayed light emission decay at times less than 120 μs, after a 10 ns excitation flash, is temperature insensitive, while for times greater than 120 μs a temperature dependent component is observed (Fig. 36). The decay kinetics of delayed light emission for times less than 20 μs is believed [15, Chapters III and IV] to be due to the removal of P^{+}_{680}, which is formed during excitation by the following reaction: $ZP^{+}_{680}Q^{-} \longrightarrow Z^{+}P_{680}Q^{-}$, where P^{+}_{680} is the oxidized form of the Photosystem II reaction center chlorophyll, Q^{-} is the reduced form of the "primary" electron acceptor, and Z is a secondary electron donor. The rise in chlorophyll a fluorescence yield following an excitation flash is a means of monitoring the above reaction [15]. As expected from the delayed light emission data (Fig. 36), the $ZP^{+}_{680}Q^{-} \longrightarrow Z^{+}P_{680}Q^{-}$ reaction (measured as fluorescence rise) also had no temperature sensitivity (Fig. 37). This temperature insensitivity was reported earlier for the green alga Chlorella by Mauzerall [45]. Temperature independent electron transport reactions have been previously observed in biological systems [152, 153] and have been interpreted to be due to electron tunneling. Although our experiments were restricted to a narrow temperature range the observed temperature insensitivity

of the $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ reaction may also reflect a tunneling process for the electron transport from Z to P^{+}_{680}. An alternate possibility for explaining the temperature insensitivity is that Z and P_{680} are located in a protein complex in such a way that they are not affected by changes in the fluidity of the lipid environment.

Arrhenius plots of the rate constant k($k = 1/\tau$, where τ is the exponential decay lifetime) for the delayed light emission component at times between 120 and 340 μs were discontinuous for bush bean and spinach chloroplasts (Figs. 38 and 40) but linear for lettuce and Alaska pea chloroplasts (Figs. 38 and 39). The discontinuous change in the Arrhenius plot might have been due to abrupt changes in the fluidity of the thylakoid membrane lipids. From ESR spectra of fatty acid spin labels incorporated in chloroplasts (Figs. 41 (a) and 42 (a)) and in water-lipid dispersions of extracted lipids (Figs. 41 (b) and 42 (b)) and from differential scanning calorimetry data from water-lipid dispersion (Fig. 43), the occurrence of discontinuous changes in membrane fluidity for bush bean chloroplasts is indicated while for Alaska pea chloroplasts it is not. Table 6 presents the temperatures at which discontinuities in delayed light emission decay constant Arrhenius plots occurred and at which membrane lipid phase transitions were indicated by electron spin resonance and differential scanning calorimetry. There is a good correlation between the occurrence of discontinuities in delayed light emission decay and changes in membrane lipid fluidity. Since large structural changes cannot occur in gluteraldehyde fixed chloroplasts [139] then changes in membrane lipids at 18 ^{o}C, indicated by electron spin resonance fatty acid spin labels [129] in fixed spinach chloroplasts (Fig. 40 (b)) indicates that large structural changes are not required for

TABLE 6

TEMPERATURE CHARACTERISTICS OF CHLOROPLASTS AND THEIR EXTRACTED LIPIDS

Temperature or range of temperatures in degrees centigrade at which a discontinuity was observed in a particular measurement. Sample preparation and measuring techniques are given in Methods.

Sample	Measurement		
	Differential Scanning Calorimetry	Electron Spin Resonance of 12 Nitroxide Stearate	Delayed Light Emission for $120<t<340$ μs
Alaska pea chloroplasts	——	none	none
Lipid-water dispersion of lipids extracted from Alaska pea chloroplasts	none	none	——
Bush bean chloroplasts	——	15	12-17
Lipid-water dispersion of lipids extracted from bush bean chloroplasts	14-19	13	——

it, but the delayed light emission decay is sensitive to the membrane lipid fluidity changes that occur even in glutaraldehyde fixed samples.

The Arrhenius plot of the delayed light emission decay constant was not affected by the presence or absence of DCMU. Thus, temperature effects on the electron flow between Photosystem II and I are not involved here. Also, the invariance of Arrhenius plot activation energy when the light generated membrane potential was eliminated by gramicidin D (Fig. 39) indicates that whatever reaction determines the kinetics of the temperature sensitive delayed light emission decay, its "activation energy" is unaffected by membrane potential. This agrees with the finding in Chapter V that delayed light emission in the microsecond range was insensitive to light and salt-jump generated membrane potential.

3. Temperature Sensitivity of Chlorophyll a Fluorescence Yield

The use of chlorophyll a fluorescence as an indicator of membrane lipid phase transitions in higher plants was not successful. Both bush bean and spinach chloroplasts had discontinuous membrane lipid fluidity changes indicated by electron spin resonance data, but chlorophyll a fluorescence in these chloroplasts uniformly decreased with increasing temperature (Fig. 44). This negative result for spinach chloroplasts had been reported earlier [151]. Also, tomato chloroplasts had no discontinuous change in chlorophyll a fluorescence as a function of temperature [131], although tomato chloroplast lipids were shown [122] to undergo a phase transition at 10 ^{o}C using fatty acid spin labels. In the algae Anacystis, Cyanidium, and Euglena, however, peaks in the chlorophyll a fluorescence as a function of temperature were observed [151] and, at least for Anacystis, this peak was associated with a membrane lipid fluidity transition [126].

We were also able to observe a peak in chlorophyll a fluorescence at 9 °C (Fig. 44) in Anacystis grown at 22 °C. It seems that chlorophyll a fluorescence may act as an indicator of membrane lipid fluidity in algae but not in higher plant chloroplasts.

4. Delipidated Chloroplast Temperature Sensitivity

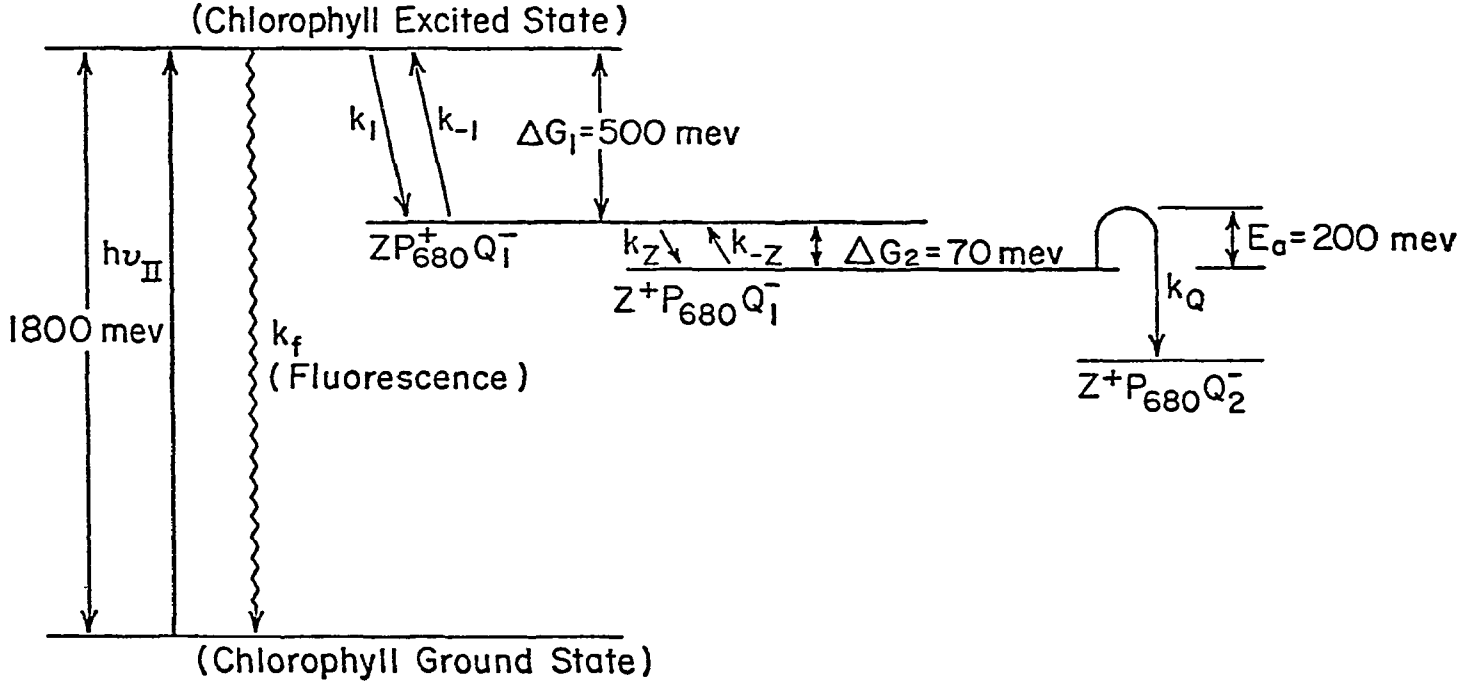
The fluorescence of tryptophyl residues in delipidated chloroplasts of bush beans and peas uniformly decreased with increasing temperature (Fig. 45). This, along with the finding that a lipid-water dispersion of extracted bush bean lipids shows a temperature discontinuity as does delayed light emission (Table 6), indicates that membrane lipids, but not proteins, are critical in determining the temperature discontinuity in delayed light emission decay at times greater than 120 μs. We interpret this to mean that the delayed light emission decay at times greater than 120 μs is sensitive to the environment in which Photosystem II is located and this environment is altered by the lipid fluidity of the thylakoid membrane.

5. Free Energy Changes Involved in Photosystem II Charge Separation: A Working Hypothesis

The arguments made here are very similar to those made by Zankel [59]; however, we include data on the 6 μs delayed light emission decay component, which he was unable to measure. Fig. 46 is a proposed energy level diagram. Upon absorption of a quantum, $h\nu_{II}$, chlorophyll goes into an excited state which may deexcite by emitting fluorescence or by transferring energy to the reaction center to cause a charge separation, $ZP_{680}^{+}Q^{-}$. Here Q has been replaced with Q_1 and Q_2 suggested in Chapter IV. The positive charge can stabilize in the $Z^{+}P_{680}Q^{-}$ form as discussed in Chapters

Fig. 46. A proposed energy level diagram of microsecond time scale Photosystem II reactions.

An energy level diagram of microsecond time scale reactions in the Photosystem II reaction center: A working model. $h\nu_{II}$ is a Photosystem II quantum, ΔG_1 and ΔG_2 are free energy changes, E_a is activation energy, k_f is the fluorescence decay rate, and all other ks are reaction rates. Z is the first secondary electron donor, P_{680} is the reaction center chlorophyll *a*, and Q_1 and Q_2 are two forms of the "primary" electron acceptor.


(Chlorophyll Excited State)
k_1
k_{-1}
ΔG_1=500 mev
hv_{II}
1800 mev
k_f
(Fluorescence)
$ZP^+_{680}Q^-_1$
k_Z
k_{-Z}
ΔG_2=70 mev
$Z^+P_{680}Q^-_1$
E_a=200 mev
k_Q
$Z^+P_{680}Q^-_2$
(Chlorophyll Ground State)

III and IV or the charges can back react to give delayed light emission. In our working hypothesis, stabilization of the negative charge can also occur by a change in the form of Q from Q_1 to Q_2 which is observable as a 50-60 μs lifetime decay component at 25 °C in NH_2OH treated chloroplasts (see proposal in Chapter IV). This same 50-60 μs component is observable in untreated chloroplasts at time greater than 120 μs when decay components due to more rapid stabilization reactions have become negligible. This reaction has a measurable activation energy of about 200 mev in pea chloroplasts (Fig. 39). The ks in Fig. 46 are the rate constants for the various reactions. The yield of delayed light emission ($\phi^{6\,\mu s}_{DLE}$) due to back reaction of P^+_{680} and Q^-_1 from the $ZP^+_{680}Q^-_1$ form is given by the following expression, assuming k_{-1} and k_{-Z} to be less than k_1 and k_Z:

$$\phi^{6\ \mu s}_{DLE} = \phi_f\left(\frac{k_{-1}}{k_{-1} + k_Z}\right)$$

where, for the 6 μs delayed light emission component, $k_Z = 1/6\ \mu s = 1.67 \times 10^5/s$ and $\phi_{DLE} = 2 \times 10^{-4}$ (calculated in Chapter IV) and $\phi_f = 2.5 \times 10^{-2}$ [73]. Using these values, k_{-1} is 1300. k_1 can be calculated by the following equation:

$$\phi_f = \frac{k_f}{k_f + k_1}$$

where, $k_f = 1/15$ ns $= 6.67 \times 10^7/s$ is the reciprocal of the intrinsic fluorescence lifetime [154]. k_1 is thus calculated to be $2.6 \times 10^9/s$ which compares well with the trapping rate of approximately $2 \times 10^9/s$ in algal cells calculated from fluorescence lifetime measurements [155]. The free energy gap between the excited state of chlorophyll a and the $ZP^+_{680}Q^-_1$ form

of the Photosystem II reaction center is, according to the Boltzmann relationship:

$$\frac{N_1 k_{-1}}{N_* k_1} = \exp(\Delta G_1 / kT)$$

where N_1/N_* is the ratio of numbers of reaction centers to chlorophyll and was found in Chapter II to be 1/200, ΔG_1 is the free energy gap, k is the Boltzmann constant, and T is the absolute temperature. Using the calculated values for the variables in the above equation, ΔG_1 is about 500 mev. Since the chlorophyll excited state corresponds to about 1800 mev of energy and ΔG_1 = 500 mev, then the $ZP^+_{680}Q^-$ form of the reaction center has stored 1300 mev of energy or about a 72% storage efficiency. This is in close agreement with the 68 to 73% theoretical efficiency for light energy capture in photosynthesis [156, 157]. The 1300 mev of energy is believed to be stored as an oxidation reduction potential difference between P^+_{680} and Q^-_1. The redox potential of Q^-_1 is uncertain, but values of -35 mV and -270 mV have been measured in chloroplasts [76] and -325 mV in Photosystem II reaction center particles [158]. The redox level of P^+_{680} has not been measured, but must be at a higher potential than the oxygen evolving S-state. Kok et al. [159] estimated its level to be >820 mV. However, using a rate constant of 10 μs for the movement of charge between P^+_{680} and the oxygen evolving complex and using a frequency factor of 10^{13}, we obtained an estimate of 1200 mV. Using the most negative value of Q^-_1 redox potential, the redox potential energy difference between P^+_{680} and Q^-_1 would be 1550 mV, and using the least negative value of Q^-_1 it would be 1235 mV. Both values are close to the 1300 mV calculated above. The yield of delayed light emission from

the 50-60 μs component can be written as:

$$\phi_{DLE}^{50\ \mu s} = \phi_{DLE}^{6\ \mu s} \left(\frac{k_{-Z}}{k_{-Z} + k_Q}\right)$$

where k_Q is the reciprocal of this reaction lifetime or 2×10^4/s and $\phi_{DLE}^{50\ \mu s} = 1 \times 10^4$ (calculated in Chapter IV). Using these values, k_{-Z} is 1×10^4/s. Applying the Boltzmann relationship, the free energy gap (ΔG_2) between the $ZP_{680}^{+}Q_1^{-}$ and $Z^{+}P_{680}Q_1^{-}$ form of the reaction center is calculated to be 70 mev. The $ZP_{680}^{+}Q^{-} \xrightarrow{k_Q} ZP_{680}^{+}Q_2^{-}$ reaction has a 200 mev activation energy (Fig. 39), but the back reaction rate constant for this reaction is not known so the free energy change for this step is not calculated.

The free energy changes in this scheme have been calculated using measured yields and decay rates of delayed light emission and are consistent with measured and estimated redox levels of Photosystem II reaction center components. Energy pooling between reaction centers is not required to explain delayed light emission, which is consistent with a one quantum process having the observed I dependence (Fig. 35).

CHAPTER VII

SUMMARY OF CONCLUDING REMARKS

The goal of this thesis was to better understand the reactions taking place in Photosystem II in isolated chloroplasts by studying the decay of delayed light emission [16] and the rise in chlorophyll a fluorescence yield [45] in the microsecond range following an excitation flash. Information on the organization of Photosystem II in the thylakoid membrane and how the membrane environment effects Photosystem II reaction were also sought. In order to make these measurements an apparatus was constructed to monitor changes beginning 6 μs after excitation. Circuits were developed to allow the rapid gating of the photomultiplier within a few microseconds after a 10 ns laser pulse. Chapter II gives details of the apparatus and experimental procedures. The major conclusions of the present study are listed in Table 7 along with the chapters, figures, and tables were supporting evidence is presented. In the following sections we briefly describe the major points.

A. The $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ Charge Stabilization Reaction

The 6 μs decay component of delayed light emission and the 6 μs rise of chlorophyll a fluorescence yield both reflect the $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ charge stabilization reaction (Chapters III and IV). Treatment of chloroplasts with TRIS or NH_2OH, which block the normal charge transfer reactions between P_{680} and the oxygen evolving system, cause a parallel inhibition of both the 6 μs component of delayed light decay and fluorescence yield rise. This 6 μs lifetime reaction was temperature insensitive in the 0 to 35 ^{o}C range (Chapter VI) suggesting that the electron flow from the donor,

TABLE 7

MAJOR CONCLUSIONS MADE IN THE THESIS

	Conclusions	Location of Supporting Evidence
1.	The 6 μs delayed light component and 6 μs fluorescence yield rise reflect the $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ charge stabilization reaction.	Chapters III and IV Figs. 5, 6, 9, 10, 11, 15, 16, 23 Tables 1-4
2.	Two charge carriers, Z_1 and Z_2, exist between the TRIS block and P_{680}.	Chapter III
3.	$MnCl_2$ donates electrons through Z_1 and not directly to P^{+}_{680}.	Chapters III and IV Figs. 7, 8 Tables 2, 4
4.	NH_2OH blocks electron flow between Z_1 and P_{680}.	Chapter IV Figs. 15, 16 Table 4
5.	The 50 to 60 μs component of delayed light is a change in the state of the "primary acceptor" Q; two states exist: Q_1 and Q_2.	Chapter IV Fig. 18 Table 4
6.	Q^{-} is essential for microsecond delayed light	Chapter IV Figs. 18, 20, 21
7.	Activation energy for microsecond delayed light is not provided by membrane potential.	Chapter V Figs. 25, 31
8.	Millisecond delayed light is enhanced by membrane potential only if a proton gradient exists.	Chapter V Figs. 27, 28, 30
9.	The light generated membrane potential after a single flash is 128 ± 10 mV (calculated using millisecond delayed light changes).	Chapter V Figs. 32, 33
10.	The $ZP^{+}_{680} \longrightarrow Z^{+}P_{680}$ charge stabilization reaction is temperature insensitive.	Chapter VI Figs. 37, 38
11.	Delayed light in the 120 to 340 μs range is temperature sensitive	Chapter VI Fig. 37

TABLE 7 (continued)

12.	Arrhenius plots of delayed light decays have discontinuities that correspond with abrupt changes in thylakoid membrane lipid fluidity.	Chapter VI Figs. 39, 40, 41, 42, 43, 44 Table 6
13.	The discontinuities correspond to microconformational changes not changes in macrostructure.	Chapter VI Fig. 41
14.	Microsecond delayed light originates from a one quantum process.	Chapter VI Fig. 36

Z, to the reaction center, P_{680}, may be by an electron tunneling mechanism. (Extension of this work to lower temperatures is necessary, however, to confirm this conclusion.)

Absorption changes at 680-690 nm have been associated with the oxidation and reduction of P_{680} [160, 161]. By measuring absorption changes after a single turn over flash in samples that had received many flashes, the reduction of P^+_{680} was observed to occur with 50 and 260 μs lifetimes [160, 161]. These absorption measurements did not reveal a 6 μs reduction rate of P^+_{680} which we observed when measuring the chlorophyll *a* fluorescence yield rise. This inability to observe a 6 μs rate in the P_{680} absorption change may be due to technical difficulties such as low signal to noise ratio and response characteristics of instruments. The 260 μs component probably reflects a recombination reaction between P^+_{680} and Q^- since Q^- decay, which is indicated by absorption change at 320 nm [162], also occurs with a 145 to 290 μs rate [162]. This type of reaction cannot be observed by changes in chlorophyll *a* fluorescence yield since both $P^+_{680}Q^-$ and $P_{680}Q$ have low fluorescence yields. A 185 to 290 μs lifetime delayed light component has been observed [47, 59] and probably is generated by this recombination reaction. We do not observe a 50 μs component in the chlorophyll *a* fluorescence yield rise that would correspond to the 50 μs P_{680} absorption data. This cannot be explained by a recombination of P^+_{680} and Q^- with a 50 μs lifetime since a 320 nm absorption change with this rate was not observed [162]. The 50 μs change in P_{680} absorption may be due to the transfer of electrons from Z_2 to Z^+_1 and P^+_{680}. This reaction has a 30 μs rate in our chloroplasts (Chapter III), but under the experimental conditions of Gläser *et al.* [161], different chloroplasts and multiflash illumination, this reaction may have a somewhat slower rate.

B. Two Charge Carriers Exist Between P_{680} and the Site of TRIS Inhibition

Two charge carrier, Z_1 and Z_2, are suggested to exist between P_{680} and the site of TRIS inhibition since the effect of TRIS washing on delayed light emission decay and chlorophyll a flurescence yield rise is not observed until two excitation flashes are given (Chapter III). This is, however, not the case for NH_2OH where its effect is observed on the first flash. Thus, NH_2OH but not TRIS, blocks the electron flow from Z_1 to P_{680}. The complex decay of microsecond delayed light may be explained by the existence of several charge carrying species having different reaction rates.

The electron donor $MnCl_2$ does not donate electrons directly to P^+_{680}, but through Z_1, since $MnCl_2$ can reverse the effects of TRIS washing but not the effects of NH_2OH on microsecond delayed light emission and fluorescence yield rise (Chapters III and IV).

C. The Primary Charge Acceptor Exists in Two Forms

The primary acceptor of charge, Q, is also suggested to exist in two forms, Q_1 and Q_2, since a 50 to 60 μs delayed light decay component persists in chloroplasts treated with NH_2OH and DCMU. Under these conditions, the known stabilization reactions for the P^+_{680} (on the water side) and Q^- (on the plastoquinone side) charges are blocked. We, thus, propose a new stabilization reaction, $P_{680}Q_1^-Q_2 \longrightarrow P_{680}Q_1Q_2^-$ with a 50 to 60 μs reaction lifetime at 25 °C (Chapter IV). In untreated chloroplasts this component is most easily seen at times greater than 120 μs after the flash. It is sensitive to temperature, and shows an activation energy of about 200 mev in lettuce and pea chloroplasts (Chapter VI). Arrhenius plots of the rate constant for this reaction have discontinuities at temperatures where the thylakoid membrane lipids undergo abrupt changes in fluidity. In plants

that have uniform changes in lipid fluidity with temperature the Arrhenius plots are linear. The sensitivity of this reaction to the state of membrane lipids may be due to Q_1 and Q_2 being quinone type molecules that are intimately associated with lipids.

D. Microsecond Delayed Light Originates from $P^+_{680}Q^-$ Charge Recombination and Is a One Quantum Process

All of the experimental results were consistent with the $P^+_{680}Q^-$ charge recombination theory for the origin of delayed light. The most important evidence is summarized here. TRIS and NH_2OH treatment of chloroplasts which caused P^+_{680} to remain higher in concentration than in control led to a ~3 fold increase in the yield of delayed light (Chapters III and IV). Addition of silicomolybdate, which competes for the charge of the primary acceptor, Q, greatly reduced delayed light emission, after a single flash, given to dark-adapted (15 min) chloroplasts had an I dependence (Chapter VI). This implies that microsecond delayed light is a one quantum process; a requirement of the charge recombination theory. Other theories of the origin of delayed light, such as electron-hole recombination [163] and triplet fusion [32] require a two quantum process. Therefore, the I dependence for microsecond delayed light makes these other theories suspect.

E. Microsecond Delayed Light is Not Affected by Membrane Potential

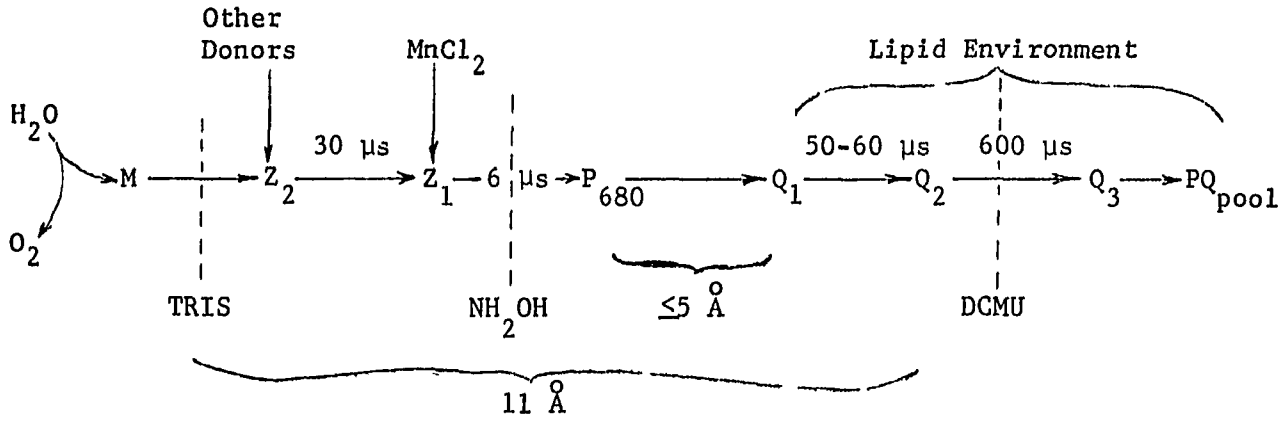
The activation for microsecond delayed light emission is not provided by the membrane potential (Chapter V) as there is no effect of either light or salt-jump generated membrane potential on microsecond delayed light intensity. This finding invalidates previous suggestions [93], which are based on measurements in the millisecond range, that delayed light would have its activation energy altered by membrane potential.

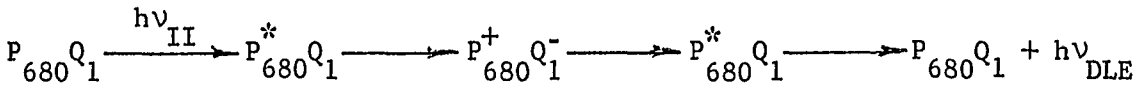
Millisecond range delayed light after a single excitation flash is enhanced by membrane potential only if a proton gradient is present (Chapter V). All previous delayed light measurements made with membrane potential as a variable were done with continuous illumination or multiple excitation. The preillumination itself generates a proton gradient; the requirement that a proton gradient be present for a membrane potential effect on millisecond delayed light to be seen was not appreciated in the earlier work. By using changes in millisecond delayed light, the light generated membrane potential, generated across the thylakoid membrane by a single excitation flash, was calculated to be 128 ± 10 mV. This is in agreement with the recent measurements of Zickler and Witt [104] based on voltage dependent ionophores.

F. A Model for Photosystem II Reactions

Finally, on the basis of analysis of microsecond and millisecond delayed light emission (Chapter V), we estimate that P_{680} and Q_1 are ≤ 5 Å apart, and Q_2 and one of the species involved in charge accumulation for oxygen evolution are ~11 Å apart. Thus, P_{680} and Q do not span the entire membrane; this contradicts the picture of Witt and coworkers [164].

In conclusion, the following scheme for the operation of Photosystem II is considered highly likely:





(This is a one quantum process)

The symbols are as previously defined with the following additions: Q_3 is a quinone labelled R or B by other authors [64, 165] and PQ is plastoquinone. The flow of charge between Q_2 and Q_3 is sensitive to DCMU and has a 600 μs lifetime in normal chloroplasts [58]. The 50 to 60 μs reaction is temperature sensitive while the 6 μs reaction is not. This picture is somewhat more complicated than the one we started with at the beginning of the present research. However, it does provide a better understanding, we believe, of Photosystem II reactions in isolated chloroplasts and of the organization of primary components in the thylakoid membrane.

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VITA

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He is a coauthor of the following publications:

1. "Thermoluminescence and Temperature Effects on Delayed Light Emission (Corrected for Changes in Quantum Yield of Fluorescence) in DCMU Treated Algae." Photochemistry Photobiology, 15, 331-348 (1972).
2. "Delayed Light Emission in DCMU Treated Chlorella: Temperature Effects." In: Proceeding of the Second International Congress on Photosynthesis Research (G. Forti, M. Avron, and A. Melandri, eds.) pp. 223-232. Dr. W. Junk, N. V. Publishers, The Hague (1972).
3. "A Major Site of Bicarbonate Effect in System II Reaction: Evidence from Electron Spin Resonance Signal II_{vf}, Fast Fluorescence Yield Changes and Delayed Light Emission." Biochimica Biophysica Acta, 440, 322-330 (1976).

4. "The Rise in Chlorophyll a Fluorescence Yield and Decay in Delayed Light Emission in TRIS Washed Chloroplasts in the 6-100 μs Time Range after an Excitation Flash." Biochimica Biophysica Acta, in press (1977).