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MEMBRANE POTENTIAL AND MICROSECOND TO MILLISECOND DELAYED LIGHT EMISSION AFTER A SINGLE EXCITATION FLASH IN ISOLATED CHLOROPLASTS

P. JURSINIC, GOVINDJEE* and C. A. WRAIGHT

Departments of Physiology and Biophysics, and Botany, University of Illinois, Urbana, IL 61801, U.S.A.

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Abstract—The effect of light-induced and salt-jump induced membrane potential on microsecond and millisecond delayed light emission from chloroplasts, following a single 10 ns flash, have been studied. Microsecond delayed light emission is shown to be independent of the membrane potential contrary to proposals that the activation energy for delayed light emission can be modulated by transmembrane electric fields. This result is discussed in terms of the possible origin of this short-lived emission. Millisecond delayed light after a single excitation flash is enhanced by membrane potential *only* if a proton gradient is present. By measuring changes in ms delayed light caused by simultaneous injection of KCl and Na-benzoate (which creates a proton gradient) in the presence of valinomycin, the light-induced potential generated across the thylakoid membrane by a single excitation flash was calibrated and found to be 128 ± 10 mV in agreement with the recent measurements of Zickler and Witt (1976) based on voltage-dependent ionophores. It is concluded that the secondary charges that give rise to ms delayed light, after a single flash, do not fully span the membrane.

INTRODUCTION

Delayed light emission, discovered by Strehler and Arnold (1951), 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 uncouplers (Mayne, 1967) and production of transmembrane pH and salt gradients strongly affect the intensity of delayed light emission in the millisecond and seconds time range (Mayne, 1968; Miles and Jagendorf, 1969; Barber and Kraan, 1970; Kraan *et al.*, 1970; Wraight and Crofts, 1971). During illumination the induction kinetics of ms delayed light emission are controlled not only by electron transport but also by the development of the light-induced proton uptake and membrane potential (Wraight and Crofts, 1971; Wraight *et al.*, 1971). These effects were ascribed to a modulation of the effective activation energy for emission by the electrical and chemical gradients of the proton motive force of the chemiosmotic coupling hypothesis (Crofts *et al.*, 1971; Fleischmann, 1971).

The effect of salt-jump or light-induced membrane potential was established only for samples that had been preilluminated with continuous light or multiple pulses associated with the phosphoroscope method (Kraan *et al.*, 1970; Wraight and Crofts, 1971). 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 (Lavorel, 1975).

All earlier studies, noted above, had used techniques which generate a proton gradient during preillumination. There is some indication (Barber and Varley, 1972) 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 μs and ms 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.

MATERIALS AND METHODS

In these experiments, chloroplasts from Alaska pea leaves 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 containing 0.4 *M* sucrose, 0.1 *M* *N*-Tris(hydroxy-methyl)methyl glycine (Tricine), 5 m*M* $MgCl_2$, 10 m*M* NaCl, and 20 m*M* ascorbate adjusted to a pH 7.8. The homogenate was strained through eight layers of cheesecloth and one layer of 10 μm mesh nylon cloth. The filtrate was centrifuged at 5000 ***g*** for 5 min to pellet the chloroplasts which were resuspended in 50 m*M* sodium phosphate buffer (pH 7.8) to obtain broken chloroplasts. These chloroplast fragments (thylakoids) were pelleted by another 5000 × ***g*** centrifugation for 5 min and were finally resuspended to a chlorophyll (Chl) concentration of 3 mg/mℓ in the grinding medium except that 50 m*M* phosphate substituted for Tricine buffer. All delayed light and Chl *a* fluorescence yield measurements were made with samples containing 5 μg Chl/mℓ. Chlorophyll concentration was determined by the method of Arnon (1949) using MacKinney's equations (MacKinney, 1941).

*All correspondence to: Govindjee, Botany Department, 289 Morrill Hall, University of Illinois, Urbana, IL 61801, U.S.A.

For salt-jump experiments, chloroplasts were treated as described by Barber and Varley (1972) and were washed twice and finally resuspended in a medium containing 5 m*M* *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (Tes) and 0.4 *M* sucrose (adjusted to a pH of 7.1 with KOH; the final concentration of KOH was 2 m*M*).

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, 400 ns) was provided by a Phase-R Model DL-1100V dye laser operated at 660 nm with a mixture of 80 μ*M* cresol violet percholate and 50 μ*M* 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. The concentration of Chl in the sample was 50 μg/m*l* of suspension.

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-in water filter, a lens, and a Kodak Wratten No. 16 orange gelatin filter; the incident light intensity at the sample was 1.3 k $W \cdot m^{-2}$.

The apparatus for measuring delayed light emission in the ms and μs ranges has already been described (Jursinic and Govindjee, 1977a; Jursinic, 1977). In all experiments the output for the nitrogen laser was about 1 mJ per pulse (at 337 nm), and the absorbed intensity equivalent to an average of about two quanta per photosynthetic unit (Jursinic and Govindjee, 1977b). 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 100 $W \cdot m^{-2}$.

RESULTS

Effects of gramicidin D on μs delayed light emission

Flash illumination of chloroplasts produces a light-generated membrane potential, ψ_l, across the thylakoid membrane (Junge and Witt, 1968). In order to demonstrate that ψ_l was being generated in our chloroplasts, the electric field indicating absorption change at 518 nm was measured beginning at 25 μs after an excitation flash (Fig. 1). The amplitude and decay time are in agreement with a range of reported results (Junge and Witt, 1968). According to estimates made for chloroplasts, based on thylakoid membrane capacitance and approximate displaced charge, this absorption change corresponds to a light generated potential of 50–100 mV (Junge and Witt, 1968). Addition of 1 μ*M* gramicidin D (1 per 50 Chl molecules) completely eliminated the 518 nm absorption change and, thus ψ_l, in our samples in the μs time range. Therefore, the μs delayed light emission, if due to charge recombination, was expected to be affected by gramicidin D in the same manner postulated to occur for ms delayed light.

It should be noted that the origin of μs delayed light emission is not clear at this time. The blockage of PS II reaction center charge separation by treatment with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and hydroxylamine eliminated delayed light in the ms and greater time range (Bennoun, 1970; Mohanty *et al.*, 1971), while in the μs range a fast component may be enhanced (Lavorel, 1973) or at least unaltered (Jursinic and Govindjee, in preparation). Microsecond delayed light is also observed when Q is kept in its reduced state by anaerobic conditions (Van Best and Duysens, 1977). Stacy *et al.* (1971) suggested that delayed light emission could originate in Chl triplet recombination, a possibility which, especially for short term emission, seems more credible in view of significant triplet formation under flash illumination (Mauzerall, 1976). Alternatively, delayed light emission with Q reduced could indicate the operation of an alternate or, perhaps, more primary acceptor such as Q_{aux} postulated by Diner (1974) or W, as suggested by Van Best and Duysens (1977).

Figure 2 shows delayed light emission decays, in the μs time range, with and without gramicidin D (up to 1 per 5 Chl molecules). Complications due to changes in the electron flow rate from Q^-, the reduced form of the "primary" acceptor of PS II, to the intersystem carrier are not expected since these decays are after a single flash. Gramicidin D had no effect on μs 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. 2).

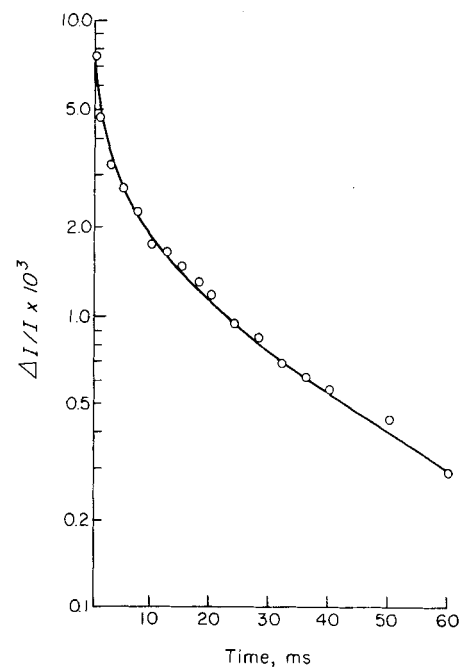

Figure 1. Logarithmic plot $\Delta I/I$ (I is the transmitted light intensity) at 518 nm from 25 μs to 60 ms after a 660 nm flash. Chlorophyll concentration, 50 μg/m*l*; optical pathlength, 1 cm; medium, 50 m*M* sodium phosphate, 400 m*M* sucrose and 50 m*M* KCl at pH 7.8.

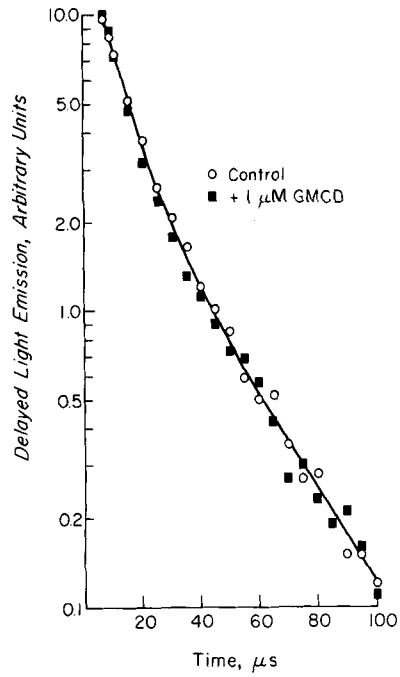

Figure 2. Logarithmic plot of delayed light emission with and without gramicidin in the 6–100 μs range after a single saturating 10 ns 337 nm flash. Chloroplasts were dark adapted for 5 min prior to excitation. Chlorophyll concentration, 5 μg/mℓ; medium, 50 mM phosphate, 400 mM sucrose, and 40 mM KCl at pH 7.8; O–O–O, control, and, ■–■–■, plus 1 μM gramicidin D (1: 5 Chl).

Contrary to the single flash data of Fig. 2, chloroplasts that had received at least ten preillumination flashes (given at a rate of 1 flash/2 s) did show an effect of 1 μM gramicidin D (1:5 Chl molecules) on μs delayed light emission (Fig. 3). However, 0.1 μM gramicidin D caused very little or no change in μs delayed light emission (Fig. 3) even though this concentration was sufficient to completely eliminate the 518 nm absorption change in this time range. Addition of 0.1 μM DCMU (DCMU to Chl = 1 to 50) inhibited μs delayed light emission to the same extent as 1 μM gramicidin D and gramicidin had no additional effect. Thus, under conditions of preillumination 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. The rather small effect of DCMU on the decay rate of delayed light in the 6–100 μs range is consistent with the fact that even without DCMU the decay of Q^- has a halftime of about 300 μs.

Effects of gramicidin D and nigericin on ms delayed light emission

The membrane potential generated by a single flash persists well into the ms time range (Fig. 1). We looked for the effects of this membrane potential on ms delayed light emission after a single flash. As shown in Fig. 4(a), 1 μM gramicidin D (1 per 5 Chl) caused no change in the ms delayed light emission following a single excitation flash. However, after preillumination, which caused a large enhancement of delayed light emission, a strong gramicidin D effect *was* observed [Fig. 4(b)] in confirmation of earlier results (Mayne, 1967; Kraan *et al.*, 1970). 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 under these conditions [Fig. 4(b)] could, therefore, be due to the loss of the light-generated membrane potential.

The enhancement of delayed light emission by preillumination (Fig. 4) was maximal with 30 s of preillumination (laser flashes at 32 Hz). 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; Shavit and San Pietro, 1967) was added; it eliminated the enhancement of delayed light emission by preillumination [Fig. 4(c)]. The addition of 1 μM valinomycin (an ionophore which specifically increases membrane permeability for K^+; Chappell and Harrhoff, 1967) only partially inhibited the preillumination enhancement of delayed

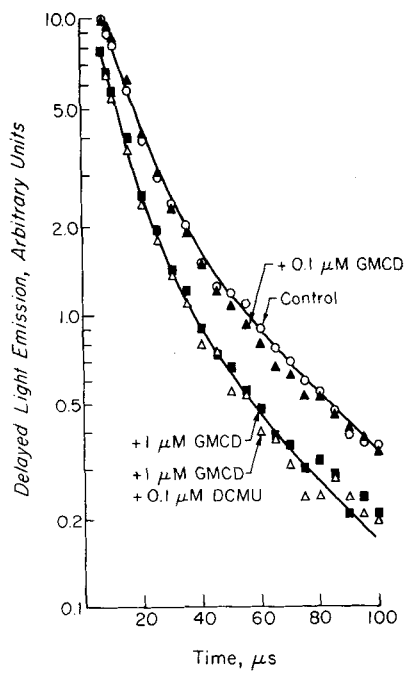

Figure 3. Logarithmic plot of delayed light emission with and without gramicidin in the 6–100 μs range following seven or more preillumination flashes given at a rate of 1 flash/2 s. Chlorophyll concentration, 5 μg/mℓ; medium, 50 mM phosphate, 400 mM sucrose and 40 mM KCl at pH 7.8; O–O–O, control; ▲–▲–▲, plus 0.1 μM gramicidin D (1: 50 Chl); ■–■–■, plus 1 μM gramicidin; and △–△–△, plus 1 μM gramicidin and 0.1 μM DCMU (1 DCMU: 50 Chl).

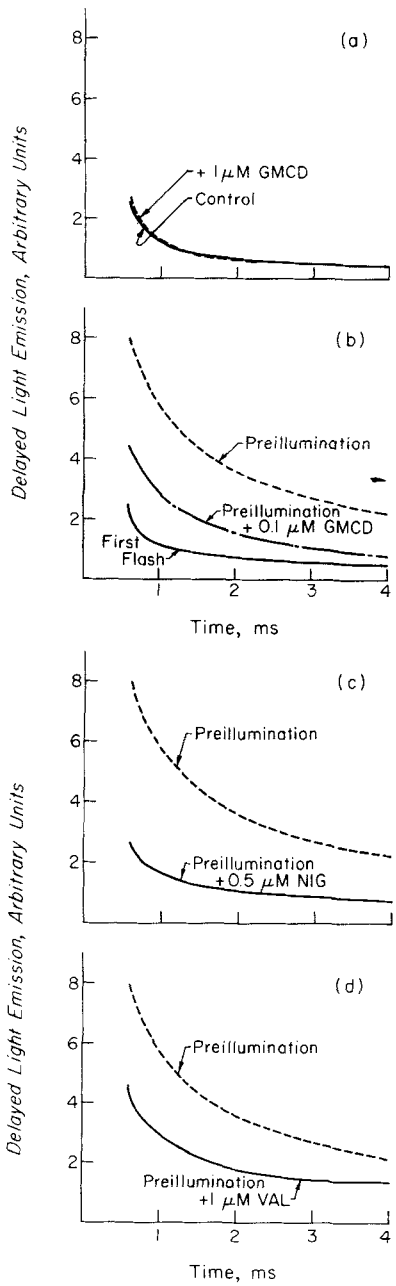

Figure 4. Delayed light emission in the 0.6–4 ms range with and without gramicidin, nigericin or valinomycin. Chlorophyll concentration, 5 μg/m/; medium, 400 m*M* sucrose and 5 m*M* TES (brought to pH 7.1 with 2 m*M* KOH); (a) after a single excitation flash with (– – –) or without (— —) 0.1 μ*M* gramicidin; (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 μ*M* gramicidin D; (c) 30 s of preillumination with laser flashes given at a rate of 32 Hz with (——) and without (– – –) 0.5 μ*M* nigericin, and (d) with (——) or without (– – –) 1 μ*M* valinomycin.

light emission [Fig. 4(d)], which is similar to the 0.1 μ*M* gramicidin D effect [Fig. 4(b)]. At these concentrations it seems that gramicidin D and valinomycin eliminate the membrane potential enhancement of delayed light emission leaving a residual enhancement which can be suppressed by nigericin.

The enhancement of delayed light emission by preillumination was related to net H^+ uptake (Fig. 5). The decay of the intensity of delayed light emission at 1.8 ms after a single flash given at various times after termination of pre-illumination was kinetically identical to the decay of the light-induced H^+ uptake.

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 (Miles and Jagendorf, 1969; Barber and Kraan, 1970). As noted in the Introduction, these earlier studies dealt with such salt-jump effects on delayed light emission only after continuous illumination or with the phosphoroscope in the ms and s range. We report here the effects of the salt-jump on delayed light emission in the μs and ms range after a single flash.

Figure 6 shows the enhancement of delayed light emission following injection of 120 m*M* KCl to preilluminated chloroplasts containing 1 μ*M* valinomycin. (Injection of NaCl caused no enhancement of delayed light emission; data not shown.) This result, which is in agreement with those of Barber and Kraan (1970), demonstrates that to see a maximal salt-jump

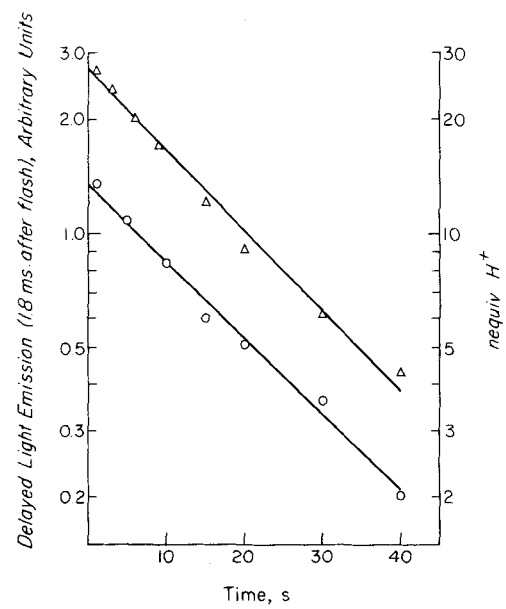

Figure 5. Delayed light intensity at 1.8 ms and external H^+ ion concentration versus time after preillumination. O–O–O, logarithmic plot of the delayed light emission intensity at 1.8 ms after a final excitation flash versus the time at which the final flash was given after the end of a 30 s preillumination period with laser flashes given at a rate of 32 Hz. Chlorophyll concentration, 5 μg/m/; medium, 400 m*M* sucrose, 40 m*M* KCl, and 50 mM sodium phosphate at pH 7.8. △–△–△, logarithmic plot of the external H^+ ion concentration versus the time after termination of 30 s continuous illumination. Chlorophyll concentration, 25 μg/ml; medium, 40 m*M* KCl at pH 7.8.

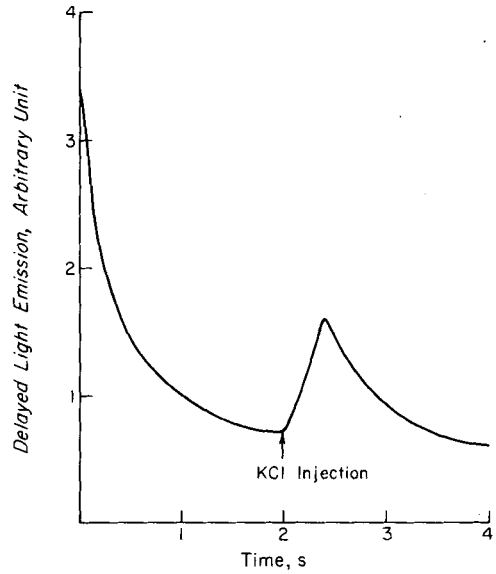

Figure 6. KCl-induced delayed light emission in the seconds range following termination of 10 s continuous illumination. Chlorophyll concentration, 25 μg/mℓ; medium, 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH) with 1 μM valinomycin; a small volume of 3 M KCl was injected at two seconds after termination of illumination to obtain a final external KCl concentration of 120 mM.

effect, under single flash conditions, the excitation flash must be given ~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 [Fig. 7(a)]. Enhancement was seen, however, following injection of sodium benzoate which establishes a proton gradient across the thylakoid membrane [Fig. 7(b)]. (See Discussion for detailed arguments.) The Na-benzoate induced enhancement of delayed light emission, after a single flash, was eliminated by 0.5 μM nigericin (nigericin:Chl = 1:10) [Fig. 7(c)]. A KCl-jump enhancement of ms delayed light emission was observed only if the chloroplasts were preilluminated; 0.5 μM nigericin also eliminated this KCl-induced enhancement. Thus, it is clear that an H^+ gradient is necessary for observing the effect of ψ_l on ms delayed light. One μM valinomycin, present in all cases, did not significantly affect the proton uptake 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. 8). Sodium benzoate injection also caused no enhancement of the μs delayed light emission (data not shown). Thus, it is unlikely that activation energy for μs delayed light is provided by a membrane potential.

Estimate of the light generated membrane potential after a single flash

An earlier attempt by Barber (1972) to calibrate the light-generated membrane potential, ψ_l, by com-

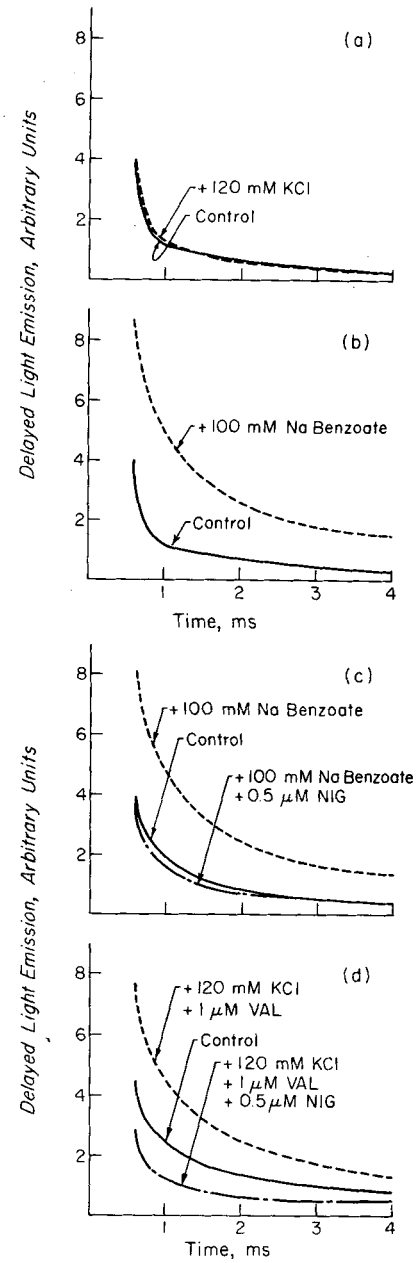

Figure 7. Delayed light emission decays after salt-jumps in the 0.6 and 4 ms range. Salt-jumps were made by injecting small volumes of 3 M salt solutions to obtain final concentrations as indicated in the figure. Except for the control (——), all decays are from a final laser flash given 0.5 s after a salt injection under the following conditions: (a) after a single flash preceded by KCl injection (– – –) to a final concentration of 120 mM with 1 μM valinomycin present; (b) after a single flash preceded by Na-benzoate injection (– – –) to a final concentration of 100 mM; (c) after a single flash preceded by Na-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 preceded by KCl injection to a final concentration of 120 mM with 1 μM valinomycin (– – –) or 1 μM valinomycin and 0.5 μM nigericin present (—·—); preillumination, 30 s of laser pulses given at a rate of 32 Hz ending 1 s prior to salt injection. Chlorophyll concentration, 5 μg/mℓ; medium, 400 mM sucrose and 5 mM TES (brought to pH 7.1 with 2 mM KOH).

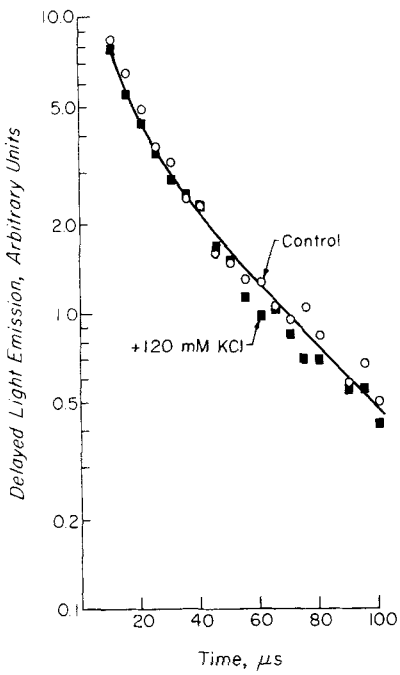

Figure 8. Logarithmic plot of delayed light emission decay in the 6 to 100 μs range following a KCl-jump. Samples were preilluminated for 30 s with laser pulses given at a rate of 32 Hz ending 1 s prior to salt injection; decays are after a single flash given 0.5 s after an injection of 120 m*M* KCl (■–■–■) or without salt injection (O–O–O). Chlorophyll concentration, 5 μg/m*l*; medium, 400 m*M* sucrose and 5 m*M* TES (brought to pH 7.1 with 2 m*M* KOH) with 1 μ*M* valinomycin present.

paring the enhancement of delayed light emission by ψ_l to that caused by salt-jump membrane potentials was made under multiple illumination conditions and, thus, is not suitable for comparison with estimates from the 515 nm absorption changes seen with single turnover flashes and, indeed, does not give good agreement with current values (Zickler and Witt, 1976). The lack of salt-jump enhancement of delayed light emission after a single flash (Fig. 7) 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 allowed a KCl-induced enhancement of ms delayed light emission to be observed after a single flash [Fig. 9(a)]. Using estimates by Barber (1972) for potassium and chloride permeabilities, a 60 m*M* KCl (final concentration) injection was calculated to induce a maximum potential of 67.1 mV. According to Crofts *et al.* (1971) $(L'/L) = \exp(F\psi_S/RT)$, where L' 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 m*M* KCl jump, $L'/L = \exp(67.1\text{ mV}/25.4\text{ mV}) = 14$. However, the experimentally determined L'/L is only 2.14 [Fig. 9(a)]. This unexpectedly low value might be explained in two ways: (1) the membrane potential which effectively alters delayed light emission is smaller than the one generated across the entire thylakoid membrane, or (2) only a certain fraction of the total delayed light emission is sensitive to membrane potential. These two possibilities are developed below.

(1) Since the recombining charge pair, giving rise to delayed light emission, may not bridge the entire thickness of the thylakoid membrane, it is not unreasonable that only part of the total potential established across the thylakoid membrane is effective in altering delayed light emission. This has been suggested for charge separation across the chromatophore membrane of photosynthetic bacteria (Jackson and Dutton, 1973; Evans and Crofts, 1974). The effective membrane potential, ψ_D, will then be given by $\psi_m \times (l/d)$, 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. 9(a), $\exp(\psi_D/25.4\text{ mV}) = 2.14$ giving $\psi_D = 19.3$ and $d/l = \psi_s/\psi_D = 67.1\text{ mV}/19.3\text{ mV} = 3.47$. The value of d/l obtained from salt-jumps at various salt concentrations was highly repeatable with an average value of 3.55 (Table 1). Since the thylakoid membrane thickness is estimated to be 40 Å (Kirk, 1971), then l is ~ 11 Å. By measuring L'/L the

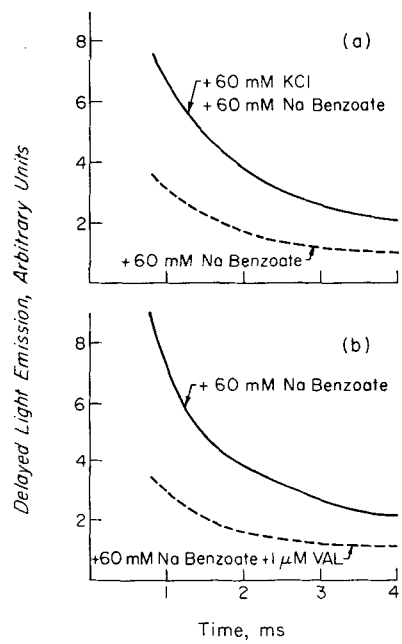

Figure 9. Delayed light emission decays in the 0.6 to 4 ms range following injection of Na-benzoate. All decays are after a single flash given 0.5 s prior to salt injection. (a) Injection of Na-benzoate to a final concentration of 60 m*M* (– – –) or simultaneous injection of sodium benzoate and KCl to a final concentration to 60 m*M* each; 1 μ*M* valinomycin was present. (b) Injection of Na-benzoate to a final concentration of 60 m*M* with (– – –) and without (——) 1 μ*M* valinomycin. Chlorophyll concentration, 5 μg/m*l*; medium, 400 m*M* sucrose and 5 m*M* TES (brought to pH 7.1 with 2 m*M* KOH).

Table 1. Salt-jump enhancement of millisecond delayed light emission

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)

Data are 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 the salt-jump (L'/L) are obtained from decay data such as in Fig. 9(a), d is the thylakoid membrane thickness, and l is the distance, normal to the membrane surface, between the recombining charges.

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 flash in the presence and absence of 1 μM valinomycin was determined. As noted earlier, the effect of ψ_l after a single flash was made observable by injection of Na-benzoate [Fig. 9(b)]. Using the formula given above, the decay in the millisecond range was calculated from Fig. 9(b), and is plotted in Fig. 10 (△–△–△). For comparison, the decay of the 518 nm absorption change is also shown in Fig. 10 (○–○–○)—the decay kinetics are similar to those of ψ_l. From the decay of ψ_l, indicated by the 518 nm absorption change (Fig. 1), maximum value for ψ_l, 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_l assumes that the substrate for delayed light emission decays at the same rate with or without valinomycin. The close correspondence between the calculated ψ_l decay and the observed 518 nm absorption change decay, therefore, suggests that the decay of these substrates (e.g. P^+, Q^-) by other routes is not strongly dependent on ψ_l.

(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 67.1 mV salt-jump generated membrane potential $L' = L_c + 14\,L_\psi$, where L_c and L_ψ are portions of the delayed light emission intensity insensitive and sensitive to membrane potential, respectively. From Fig. 9(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 sensitive to the potential varies with the value of ψ_s used as the starting point for this calculation, thus making this approach suspect. Nevertheless, ψ_l was calculated from the data of Fig. 9(b) and plotted in Fig. 10 (●–●–●). The decay rate of ψ_l, 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_l on delayed light emission less satisfactory than the former [(1) above].

Addition of ferricyanide was used to diminish PS I activity by chemically oxidizing P_{700}, the PS I reaction center, in the dark. Complete blockage of PS I activity has been reported by Schliephake *et al.* (1968) to decrease the 515 nm absorption change by 50%. In our chloroplasts, 5 mM ferricyanide reduced the 518 nm absorption change by 44% and ψ_l, calculated using the delayed light emission method described under (1) above, by 49%. As expected, lower concentrations of ferricyanide caused only partial blockage of PS I activity and smaller decrease in membrane potential. For 0.05 mM ferricyanide the 518 nm absorption change was reduced by 28% and the membrane potential, calculated by delayed light emission, by 20%.

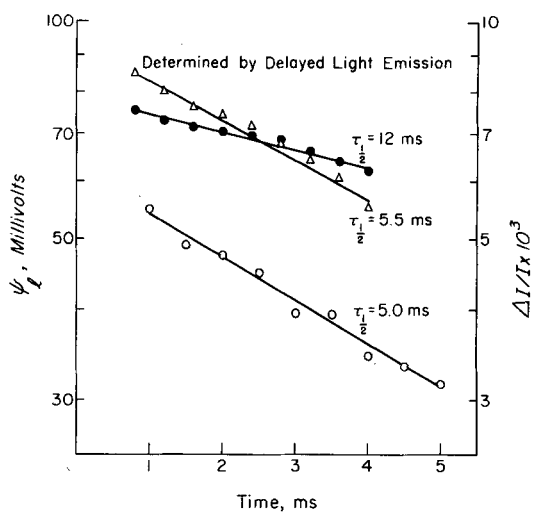

Figure 10. Logarithmic plot of the light-generated membrane potential, ψ_l, determined by salt-jump enhanced delayed light after a single flash. Two methods for calculations of ψ_l from delayed light were (1) by assuming that only a portion of ψ_l is effective in altering the delayed light emission intensity (△–△–△) and (2) by assuming that only a portion of the total delayed light emission intensity is sensitive to ψ_l (●–●–●). The logarithmic plot of the 518 nm absorption change ($\Delta I/I$), after a single flash, is also shown (○–○–○).

DISCUSSION

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 μs or ms delayed light emission is seen after a single excitation flash [Figs. 2, 4(a) and 7(a)]. With preillumination, however, enhancement of delayed light in the ms range by light generated membrane potentials and KCl jump diffusion potentials are observable [Figs. 4(b) and 7(d)]. The requirement for preillumination may arise from a dependence on the light-generated proton gradient across the thylakoid membrane or a shift in the internal thylakoid pH. Whatever the origin, the effect is shown by the elimination of the preillumination enhancement of delayed light emission by nigericin [Fig. 4(c)], similar decay kinetics of the preillumination enhancement of delayed light emission and the H^+-ion efflux measured with a pH electrode (Fig. 5) 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, most previous results on light generated membrane potential and salt-jump diffusion potential effect (Mayne, 1967; Miles and Jagendorf, 1969; Barber and Kraan, 1970; Barber and Varley, 1972; Barber, 1972) were obtained with techniques that involved preillumination of the sample; thus, a requirement for preillumination and proton gradient would not be appreciated. However, Joliot and Joliot (1974) observed in algae that ms delayed light and PS I generated membrane potential had parallel increases after only two short, saturating flashes. A pH gradient would thus appear not to be a prerequisite for observing enhancement by a membrane potential. However, the true status of transmembrane gradients is unknown in intact cells. Alternatively, the controlling factor may be thylakoid internal pH (also unknown in whole cells) rather than the pH gradient. Internal pH has been shown to affect both ms (Wraight *et al.*, 1972) and μs (Haveman and Lavorel, 1975) delayed light. Enhancement of the ms emission at low pH was correlated to inhibition of the S-state cycle and could be explained by inhibition of a charge transfer step on the O_2-evolving side of PS II and the accumulation of a higher S-state (Wraight *et al.*, 1972; Van Gorkom *et al.*, 1976). A requirement for low internal pH could also suggest that ms delayed light originates from the back reaction of Q^- with a protonated form (ZH^+) of Z as proposed by Kraan *et al.* (1970).

Using the phosphoroscope method, Wraight and Crofts (1971) observed valinomycin-sensitive enhancement of the fast induction phase of ms delayed light even in the presence of nigericin and attributed this to an effect of the membrane potential alone. Again, the use of repetitive illumination renders comparison difficult. Ellenson and Sauer (1976), however, have shown ms delayed light to be sensitive to an applied electric field following even single flashes. This is the only clear example in conflict with our findings and remains unresolved at present.

The effects of sodium benzoate

In previous salt-jump experiments, Na-benzoate was noted to cause a much greater stimulation of emission than other sodium salts (Barber and Kraan, 1970; Kraan *et al.*, 1970). Crofts has suggested (see Kraan *et al.*, 1970), on the basis of work on anion uptake in chloroplasts (Crofts *et al.*, 1967), that benzoate can penetrate the thylakoid membrane in the protonated form and thereby generate a pH-gradient. Such a behaviour is well established for other anions in both chloroplasts (Crofts *et al.*, 1967) and mitochondria (Chappel and Crofts, 1966). In our experiments, (i) salt-jump enhancement of delayed light emission can be observed after a single excitation flash if Na-benzoate is present [Fig. 7(b)]; (ii) injection of sodium benzoate allows the effects on delayed light emission of the light-generated membrane potential to be observed after a single excitation flash [Fig. 9(a) and (b)]; and (iii) nigericin eliminates both these effects of Na-benzoate. These results are consistent with the interpretation that Na-benzoate establishes a proton gradient across the thylakoid membrane.

The light-generated thylakoid membrane potential after a single flash

The ability of Na-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 Na-benzoate and KCl followed by a single flash allowed the enhancement of delayed light emission by a known diffusion potential to be established [Fig. 9(a)]. The effect of the light-generated membrane potential after a single flash, was also observable following injection of Na-benzoate (Fig. 9(b)) and was used to estimate a value for the initial light-generated membrane potential of 128 ± 10 mV with decay kinetics in the ms range similar to those seen for 518 nm absorption changes (Fig. 10). The magnitude of 128 mV agrees well with recent estimates of 105–135 mV based on voltage-dependent ionophores (Zickler and Witt, 1976), but is higher than earlier estimates of 50 mV based on the 518 nm absorption change (Junge and Witt, 1968; Schliephake *et al.*, 1968) and 30–70 mV determined with micro-electrodes placed across an intact chloroplast (Vredenberg *et al.*, 1973; Vredenberg and Bulychev, 1976).

Inhibition of PS I activity by 5 m*M* 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 ms delayed light emission is influenced by the membrane potential.

The method of calculating the membrane potential from the ms delayed light emission suggests that the distance between the recombining charges is only about 11 Å perpendicular to the thylakoid membrane surface. Quantitatively, this result rests on the assumption of a homogeneous dielectric and is thus open to criticism, but the qualitative conclusion of incomplete spanning of the membrane is independent of this assumption. At first sight, this seems difficult to reconcile with the rapid protolytic reactions occurring at both donor and acceptor sides of PS II. Ausländer and Junge (1974, 1975) have shown that the intrinsic rates of these reactions are fast ($>10^3\ s^{-1}$) but a diffusion barrier limits accessibility of the acceptor side protolysis to the external bulk phase. Such a barrier could account for the incomplete spanning of the membrane by the charge separation in the time range of a few ms. Bearing in mind, however, that Ausländer and Junge's work was performed with considerable signal averaging and thus represents a measure of the average turnover of PS II, an alternative interpretation can be found in recent work on single flash proton binding studies (C. F. Fowler; S. Saphon & A. R. Crofts, unpublished) which show that H^+-ions are released into the internal phase at each step of the oxygen-evolving cycle except for $S_1 \rightarrow S_2$. This is, in fact, the dominant process on the first flash since S_1 is the stable, dark-adapted form. Furthermore, H^+-binding from the outer phase occurs predominantly on even flashes due to a two-electron gating function on the acceptor side. Our result may, thus, suggest that for the $S_1 \rightarrow S_2$ transition, at least, the charge separation does not span the membrane in the ms time range. Different membrane-potential dependences of the delayed light emission might, therefore, be expected with flash number and could provide information on the relative dispositions of the stored charges in the S-state cycle.

It should be emphasized that regardless of the reason for the low proportionality between the delayed light intensity and the membrane potential, the calibration is internally consistent over a wide range of KCl concentrations (Table 1) and does not significantly affect the calculated value of the light-induced membrane potential.

The lack of a membrane potential effect on microsecond delayed light

The lack of effect of membrane potential on μs delayed light emission leads to the conclusion that in the μs range the thylakoid membrane potential does not provide the activation energy for delayed light emission. In the ms 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 (Crofts *et al.*, 1971).

The origins of the various components of μs delayed light are uncertain. Lavorel (1973, 1975) and Jursinic and Govindjee (1977a) have suggested that the component of 6 μs lifetime is associated with an electron transfer step from donor Z to P_{680}^+, but other results may be more compatible with a non-electron transfer process (Jursinic and Govindjee, in preparation) such as carotenoid triplet formation and decay. Also, recent results by Van Best and Duysens (1977) and Mathis *et al.* (1976)* suggest that the Z to P_{680}^+ charge transfer occurs in about 1 μs or less. A component of 50–60 μs lifetime has been associated with a change in the state of Q^- (Lavorel, 1973; Jursinic and Govindjee, 1977b) and charge recombination. The lack of a membrane potential effect on μs delayed light leads to quite different interpretations depending on the origin of the delayed light. If it originates from a process not involving charge recombination, an effect of membrane potential is not necessarily expected, whereas some effect would be likely if a charge separation and recombination were involved (Crofts *et al.*, 1971). However, if the charge separation in this time range were only over a small transverse membrane distance (≤5 Å) a 70 mV salt-jump potential would cause only a 20% enhancement, which would be barely discernible above the μs emission noise level.

Despite the lack of certainty of the origin of the delayed light emission component, the possibility of a small initial charge separation is consistent with a recent suggestion by Joliot and Joliot (1976) for PS II reaction centers.

A possible membrane model for Photosystem II

Figure 11 shows a current working model for PS II components in the thylakoid membrane that is consistent with the data presented in this paper (see e.g. Joliot and Joliot, 1976). The notion of sequential electron transfer to span the membrane is inconsistent

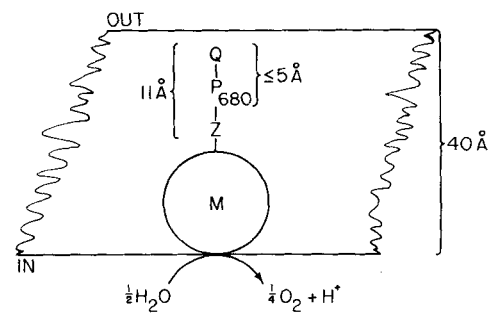

Figure 11. A current working model for the possible arrangement of PS II components in the thylakoid membrane (see also, Trebst, 1974; Joliot and Joliot, 1976). P_{680} is the reaction center Chl and the primary electron donor, Q is the "primary" electron acceptor, Z is the first secondary electron donor, and M is the chemical species which accumulates four positive charge equivalents before reacting with water to evolve oxygen. The distances are the resultants in the direction normal to the membrane and a linear arrangement is not necessarily implied.

*See note added in proof.

(without additional assumption) with the simple, nanosecond or faster, rise kinetics reported for the 518 nm absorption change (see Witt, 1975). This discrepancy is currently unresolved, but the recent data of Joliot and Joliot (1976) also seem inconsistent with the earlier ns data. The placement of Q on the outer side of the membrane is based on experiments with silicomolybdate (Zilinskas, 1975). The placement of P_{680} closer to the outside of the membrane was suggested by Arntzen *et al.* (1974) on the basis of their iodination experiments (also see Trebst, 1974; Babcock and Sauer, 1975). 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 (1974).

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Note added in proof:

When isolated chloroplasts were suspended in the medium used in our studies, P. Mathis, T. Wydvzynski and Govindjee (personal communication) indeed observed av 6 μs half time for the reduction of P^{+}_{680} by Z confirming the suggestion of Jursinic and Govindjee (1977a).