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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

P JURSINIC and GOVINDJEE*

Department of Physiology and Biophysics, University of Illinois, Urbana, Ill 61801 (U S A)

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SUMMARY

Parallel measurements of the rise in chlorophyll *a* fluorescence yield and delayed light emission decay, after a 10 ns saturating excitation flash, have been made in tris(hydroxymethyl)aminomethane-washed chloroplasts Various electron donor systems (Mn^{2+}; ascorbate; reduced phenylenediamine and benzidine) were used in conjuction with different preillumination regimes to alter [P^+-680], the oxidized form of the Photosystem II reaction center chlorophyll *a*. Conditions giving rise to high [P^+-680] resulted in only a small rise in fluorescence yield, an inhibition of a 6 μs delayed light component, and an enhancement of a 60 μs component of delayed light emission. These results confirm the hypothesis that P^+-680 acts as a quencher of fluorescence and that delayed light emission in the microsecond time range is due to the back reaction of P^+-680 and Q^-. (Q is the first "stable" electron acceptor of Photosystem II.) Two preillumination flashes are required before the full effect of Tris washing is observed in the delayed light emission decay and fluorescence yield rise; this suggests that a capacity to hold two charges exists between the Tris block and P^+-680. Tris washing has no direct effect on the movement of electrons from Z (the first electron donor to P^+-680) to P^+-680 Finally, Mn^{2+} donates electrons to P^+-680 via Z.

INTRODUCTION

The reaction center complex of Photosystem II uses the excited singlet state energy of a chlorophyll *a* molecule to generate a charge couple. This reaction is believed to take place in the following schematic form [1, 2]

$$ZP\text{-}680\ Q \xrightarrow{h\nu} ZP^*\text{-}680\ Q \rightarrow ZP^+\text{-}680\ Q^-,$$

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

* To whom reprint requests should be addressed at the Department of Botany, 289 Morrill Hall, University of Illinois, Urbana, Ill 61801, U S A

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^- [3, 4].

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 [5]. Hydroxylamine, which is believed to inhibit the $Z\,P^+$-680 $\rightarrow$ Z^+ P-680 reaction, increases the amplitude of a delayed light component with a half time ($\tau_{\frac{1}{2}}$) of 35 μs [6], while conditions designed to maximize the concentration of Z^+ stimulate a component with $\tau_{\frac{1}{2}}$ of 120 μs [7]. 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 [8, 9]. A low fluorescence yield at 16 μs after a flash corresponded with an increased amplitude of a 20 μs component of delayed light emission [10].

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 [11], incubation with chaotropic agents [12], Tris washing [13], and hydroxylamine treatment [14, 15]. 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 [16]. Furthermore, addition of exogenous electron donors ($MnCl_2$, ascorbate, phenylenediamine and benzidine) to Tris-washed chloroplasts has been used to alter Photosystem II reactions.

Delayed light emission was observed after a single saturating excitation flash instead of by the phosphoroscope method The advantages of this method are: (1) stabilization steps occurring as early as 6 μs can be observed since the excitation lasts only a few nanoseconds instead of tens of microseconds or longer in the phosphoroscope; (2) decays, after a single excitation, are not complicated by decay from previous cycle excitation; and (3) there is freedom in giving various preillumination regimes.

In addition to delayed light emission decay, we have measured in parallel the rise in chlorophyll *a* fluorescence yield in the microsecond time range after a saturating flash in isolated chloroplasts. Since the fluorescence yield rise is related to the concentration of P^+-680 [9, 17] its measurement enabled us to monitor how various treatments 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. [10] for intact algal cells 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 the first strong experimental evidence for the P-$^+$680 Q^- recombination hypothesis for microsecond delayed light emission from isolated chloroplasts

MATERIALS AND METHODS

Alaska peas (*Pisum sativum*) and bush beans (*Phaseolus vulgaris*) were grown in the laboratory and harvested 10 days after seed germination. The leaves were rinsed in ice water and then homogenized for 20 s in a Waring blender in 150 ml of buffered medium (0.4 M sucrose, 0.1 M *N*-tris(hydroxylmethyl)methylglycine (Tricine), 5 mM $MgCl_2$, 0.01 M NaCl, and 20 mM ascorbate, pH 7.8). For bean chloroplasts, 250 mg of bovine serum albumin was also added to the grinding media. The homogenate was strained through eight layers of cheesecloth and one layer of 10 μm mesh nylon cloth. The filtered liquid was centrifuged at $5000 \times g$ for 5 min to pellet the chloroplasts which were resuspended in a 50 mM phosphate buffer (pH 7.8) to obtain broken chloroplasts. These chloroplasts were pelleted by another $5000 \times g$ 5 min centrifugation and finally resuspended to a chlorophyll concentration of approx. 3 mg/ml in a solution identical to the grinding media except with 50 mM phosphate instead of Tricine as the buffer. For delayed light emission and fluorescence yield rise measurements, the chloroplasts were further diluted with the phosphate buffer to a final chlorophyll concentration of 5 μg/ml.

Tris washing of chloroplasts was carried out as described by Blankenship and Sauer [18] in 0 8 M Tris, pH 8.0, at 0 °C for approx. 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 Spring, Instrument Clarke 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 [19] to determine their oxygen evolution capacity under flash illumination conditions.

The apparatus used in measuring delayed light emission and fluorescence yield rise has been previously described [20] Both measurements were made after a single saturating flash preceded by various preillumination conditions. For both types of measurements, the actinic pulse was provided by an Avco Everett Model C102 nitrogen laser having an emission wavelength of 337 nm with a pulse width at half maximum of 10 ns. The flash intensity was adjusted to be just saturating This intensity was found to be identical for both delayed light emission and fluorescence yield changes. The fluorescence yield rise was measured by a method similar to that of Mauzerall [21]. 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. The fluorescence yield was calculated as described earlier [20]: $\Phi_f(t)$ (in terms of Φ_0) = $(F_m(t) - I_{dle}(t))/F_0$, where $\Phi_f(t)$ is the fluorescence yield at time t, $F_m(t)$ is the measured fluorescence signal at time t, $I_{dle}(t)$ is the intensity of delayed light emission at time t, F_0 is the 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.

Analog signals from the photomultiplier were digitized by a Biomation Model 805 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 then printed by teletype in numerical form for further analysis.

The delayed light emission decay was not recorded for $\tau < 6$ μs since an artifactual signal, with an exponential decay time of approx 0 8 μs, was observed in this time range. It is believed that this artifactual signal is generated by the intense burst of fluorescence from the excitation pulse causing 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 the flash and recording of the delayed light emission decay began at this time.

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 approx. 10 % 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.

RESULTS

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. Typical delayed light emission decays after the final flash of a series of flashes given every 0.5 s are shown in Fig. 1. The component lifetimes and amplitudes resolved from these semilogarithmic plots are shown in Table I, lines 1 and 2. Tris washing had a significant effect both on the 6- 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 2.

Delayed light emission decay and fluorescence yield rise 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 [10, 22] and damped out by the ninth flash. The delayed light components at $\tau < 30$ μs were found not to oscillate with period 4 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

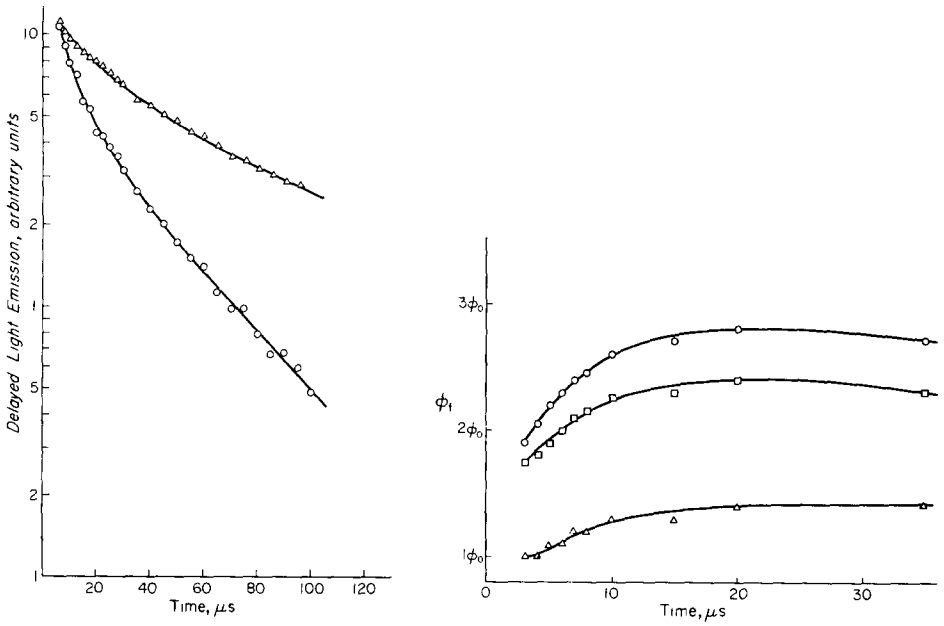


Fig 1 Logarithmic plot of delayed light emission decay from 6 to 100 μs after the final flash of a series of flashes given at a rate of 2 flashes/s Bush bean chloroplasts ○–○, control, △–△, Tris washed

Fig. 2 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 (○–○) and Tris washed (△–△), excitation rate, 1 flash/5 s· Tris washed (□–□)

TABLE I

DECAY CHARACTERISTICS OF DELAYED LIGHT EMISSION IN THE 6–100 μs TIME 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 $\pm$ 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	τ_1 (μs)	α_2	τ_2 (μs)	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

Tris-washed sample in Fig. 1. 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 I). 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 I.

In control chloroplasts, the delayed light emission decay and fluorescence 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. 2. When the fluorescence yield rise is plotted as an exponential*, the rise time of the control is approx. 6 μs. 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 approaches control; see Fig. 2 Excitation flash rates with periods between 0 5 and 5 s gave the fluorescence yield rises intermediate to those shown in Fig. 2.

Tris-washed chloroplasts with various electron donors

Mn^{2+} donation According to interpretations of electron spin resonance data [16] 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 3 and Table II. The addition of 10^{-7} M $MnCl_2$ ($MnCl_2$: chlorophyll, 1 : 50) appears to reverse the changes in delayed light emission decay caused by Tris 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. (Compare lines 1–3 of Table II). It is believed (see later) this reflects Mn^{2+} donating electrons to *P*-680 via Z. With the addition of 10^{-5} M ethylenediamine tetraacetic 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. 3) 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 II, lines 2–4. When higher concentration of $MnCl_2$ (1 $MnCl_2$ per 5 chlorophyll molecules, as in ref 16) was used, the same changes in the delayed light emission decay were observed as with lower concentrations.

Measurements of the rise in fluorescence yield after the final excitation flash under these conditions are shown in Fig. 4. 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 after two flashes is interpreted (see Discussion) to mean that two charge carriers must exist between the site of Tris blockage and *P*-680 and these charge

* 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 exponential rise lifetime

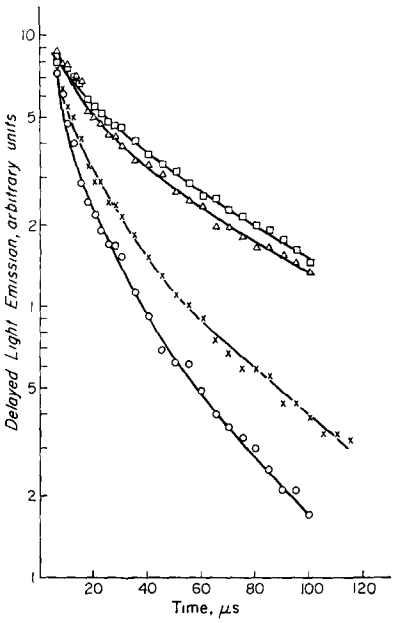


Fig. 3 Logarithmic plot of delayed light emission decay from 6 to 100 μs after the final flash in a series of flashes given at a rate of 1 flash/s. Alaska pea chloroplasts, control (○–○), Tris washed (□–□), Tris washed plus 10^{-7} M $MnCl_2$ ×–×, and Tris washed plus 10^{-7} M $MnCl_2$ and 10^{-5} M EDTA (△–△)

carriers must be oxidized to see the 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 the charge carriers.

Ascorbate and ascorbate plus phenylenediamine. In Tris-washed chloroplasts, ascorbate and ascorbate plus phenylenediamine were also used as electron donors to

TABLE II

DECAY CHARACTERISTICS OF DELAYED LIGHT EMISSION IN THE 6–100 μs TIME RANGE FOR TRIS-WASHED SAMPLES PLUS $MnCl_2$, PLUS ASCORBATE AND ASCORBATE WITH PENYLENEDIAMINE

Other information as in the legend of Table I

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 flashes/s	40	7	60	60	2 6
7	Tris washed, 10^{-4} M ascorbate, 20 flashes/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

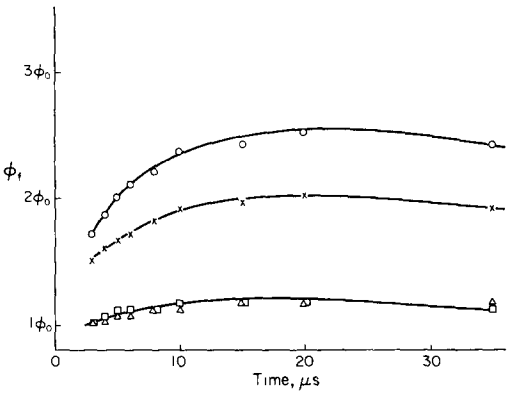


Fig 4 Plot of the rise in chlorophyll *a* fluorescence yield after a final flash in a series of flashes See legend of Fig 3 for details.

Photosystem II. Comparison of lines 1, 2 and 5 in Table II 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 II). 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 II.)

A similar dependence on flash rate was seen in the effect of ascorbate on the fluorescence rise in Tris-washed chloroplasts. Thus at 1 flash/s ascorbate alone gave fluorescence rise kinetics similar to the control, whereas, with 20 flashes/s the presence of ascorbate in Tris-washed samples had no effect on the fluorescence yield rise which reached only $1.1\phi_0$ at 20 μs after the flash. However, with ascorbate plus phenylenediamine, the Tris-washed chloroplasts exhibited a rise in fluorescence yield of 2.8 ϕ_0 at 20 μs after the flash.

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 fluorescence 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 [16].

TABLE III

DECAY CHARACTERISTICS OF DELAYED LIGHT EMISSION IN THE 6–100 μs TIME RANGE FOR TRIS-WASHED SAMPLES PLUS BENZIDINE WITH ASCORBATE

Other information as in the legend of Table I

Line	Sample conditions, bush bean chloroplasts	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

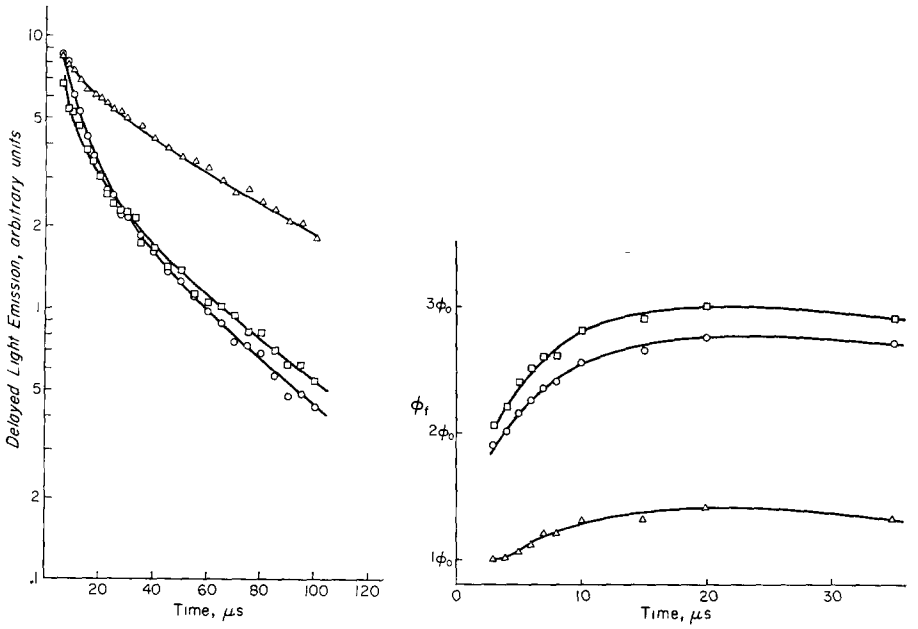


Fig 5 Logarithmic plot of the decay in delayed light emission after a final flash following a series of preillumination flashes· ○–○, untreated control chloroplasts with preillumination flashes at a rate of 1 flash/s, Δ–Δ, Tris-washed chloroplasts, 1 flash/s, and □–□, Tris-washed chloroplasts with 10^{-3} M ascorbate plus 10^{-5} M benzidine, 20 flashes/s

Fig 6 Plot of the rise in chlorophyll *a* fluorescence yield (Φ_f) in terms of Φ_0, the level of fluorescence yield prior to excitation See legend of Fig 5 for other details

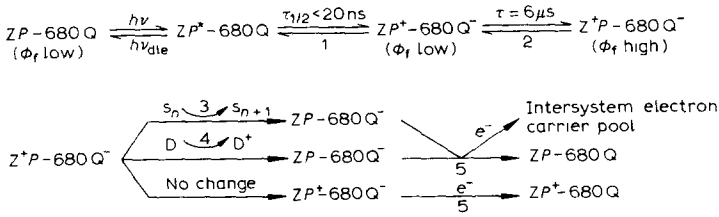
Ascorbate plus benzidine caused the amplitudes of the 6-μs component to approach that of the control, while the amplitude and lifetime of the slower component were also reduced to those of the control. (See lines 1–3 of Table III and Fig. 5.) It should be noted that this occurred even with high flash repetition rates (20 flashes/s).

The effect of ascorbate and benzidine on the rise in fluorescence yield of Tris-washed chloroplasts is shown in Fig. 6. 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.

Area under the curve. The area under the delayed light decay curve due to the components observed is shown in Tables I–III. 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 2 to 3 times greater than for the control.

DISCUSSION

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



where Z and Q are the first secondary electron donor and the first stable acceptor for Photosystem II, respectively, P^*-680 is the primary electron donor and the reaction II chlorophyll *a*, S_n and S_{n+1} represent charge accumulating sequential states of the oxygen evolving system and D is a secondary electron donor, 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 2. 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^+ [16]. 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 [9, 10, 20]. 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 $\leqslant$ 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 halflife for Z^+ reduction under various experimental conditions [16]. With a flash repetition rate of $\geqslant$ 1 Hz Z^+ P^+-680Q^- rapidly builds up and fluorescence is quenched However, as can be seen in Fig 2, 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 *P*-680Q by reactions 4 and 5 Z.

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. 4 and 6). 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 by 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 [16] 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 II, line 6).

The observed low fluorescence yield in Tris-washed chloroplasts given continuous illumination [13, 23, 24] 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 Z *P*-680 Q. As a result Z^+P^+-680Q and Z^+P^+-680Q^- 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^+-680Q^- which will augment recombination giving rise to more intense delayed light emission (see Fig. 1 and lines 1 and 2 of Table I) 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 I). This is expected since during the 5 s between flashes Z^+P-680Q^- 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. 5 and lines 5 and 8 of Table II and 3 of Table III). 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 II. 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 7). Tris washing decreases the 6 μs delayed light and the fluorescence rise components (Fig. 7b). 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 delayed 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^+-680Q^- 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^+-680Q^- 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

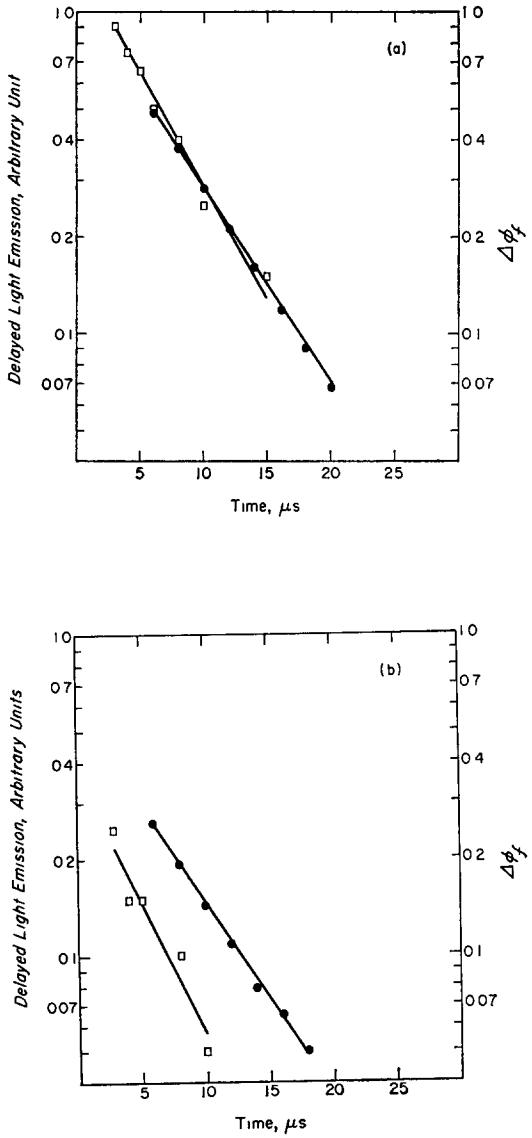
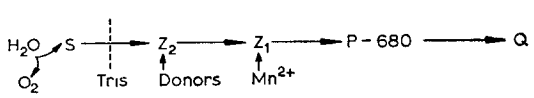
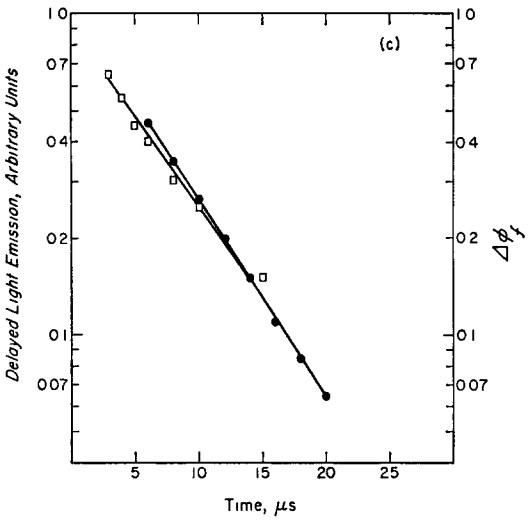


Fig 7. See opposite page for legend

call it Z_1). Due to differences with other donors (ref. 16), 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:





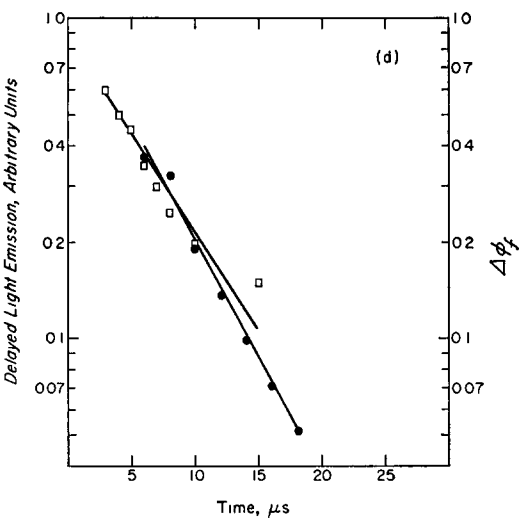


Fig 7. Logarithmic plot of the most rapidly decaying component of delayed light emission beginning at 6 μs after the excitation flash and the variable fluorescence yield, $\Delta\Phi_f = \Phi_f(\max) - \Phi_f(t)$, beginning at 3 μs. The delayed light emission decay component was obtained from decay curves 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.

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 [25, 26]. Contrary to this, Velthuys and Amesz [27], 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 after 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. 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 $\rightarrow$ $Z_1{}^+$ *P*-680 reaction completely blocked (unpublished data).

In a recent paper [28], the delayed light emission in the 0.2–20 μs time range has been studied in intact cells of *Chlorella* following short saturating flashes. A decay component of approx 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 $\rightarrow$ Z^+P-680 reaction in *Chlorella* In our chloroplast preparations we observed a 6 μs component, which correlates with a 6 μs rise of the fluorescence yield, and we believe reflects ZP^+-680 $\rightarrow$ Z^+P^+-680 reaction in chloroplasts. It is felt these differences in results may lie in the differences between the intact cells and chloroplasts. For example, differences in the relaxation time of the oxygen evolving S-states, in intact cells and chloroplasts, are known to exist [29].

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

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