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# DELAYED FLUORESCENCE FROM BACTERIOCHLOROPHYLL IN *CHROMATIUM VINOSUM* CHROMATOPHORES

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## SUMMARY

Delayed fluorescence from bacteriochlorophyll in *Chromatium vinosum* chromatophores was studied at room temperature and under intermittent illuminations.

The decay of delayed fluorescence was constituted of two components; a fast component decayed with a half time of about 8 ms, a slow one decayed in parallel with the reduction of photooxidized bacteriochlorophyll ($P^+$) with a half time of 100–200 ms. The biphasic decay of delayed fluorescence indicated that a rapid equilibrium was established between the primary electron acceptor and the secondary acceptor.

In the presence of *o*-phenanthroline, the time course of the decay of delayed fluorescence was identical with that of the reduction of $P^+$ in reaction center-rich subchromatophore particles, although they did not necessarily coincide with each other in "intact" chromatophores.

The intensity of the slow component was increased and the decay was accelerated at basic pH values. Reagents that dissipate the proton gradient across the chromatophore membranes such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and nigericin accelerated the decay of the slow component. These effects are probably resulting from changes in internal pH of chromatophore vesicles. Reagents that dissipate the membrane potential such as CCCP and valinomycin decreased the intensity.

## INTRODUCTION

Long-lived luminescence from photosynthetic systems was found in 1951 by Strehler and Arnold [1] in green plants and in 1956 by Arnold and Thompson [2] in a photosynthetic bacterium *Rhodospirillum rubrum*. The "delayed fluorescence" from photosynthetic bacteria had a similar spectrum to that of the "prompt fluorescence" from bacteriochlorophyll [2–4]. Successive investigations on photosynthetic bacteria

Abbreviations: A and $A^-$, the primary electron acceptor in its oxidized and reduced states; BChl and BChl*, bacteriochlorophyll in its ground and excited singlet states; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; *P* and $P^+$, primary electron donor in the photochemical reaction center in its reduced and oxidized states.

[4–6] established interpretation of the delayed fluorescence from bacteriochlorophyll as a result of the reversal of the primary process in photosynthesis. From thermodynamic consideration Ross and Calvin [7] pointed out that in photochemical systems luminescence due to the back reaction of primary photochemical events is expected when light energy is conserved into work with a considerable yield.

The intensity of the delayed fluorescence was stimulated by the high energy state of photosynthetic membranes [8–11]. It was suggested [10, 12, 13] that the charge separation in the primary photochemical reaction took place across the membrane and that electrical potential gradient caused a change in the activation energy for the charge recombination process.

In spite of their importance and much interest, investigations on the delayed fluorescence from photosynthetic bacteria have been far fewer than those on higher plants, and almost none of the investigations included analysis of the decay of luminescence. There have been no investigations about the decay of delayed fluorescence from bacteriochlorophyll in continuous light and at room temperature. Analysis of steady states under these conditions, however, is important for understanding energy transduction in photosynthesis under physiological conditions.

In photosynthetic bacteria, magnesium protoporphyrin, the precursor of biosynthesis of bacteriochlorophyll, also emits long-living luminescence, the lifetime of which is 20–40 ms under anaerobic conditions [14]. There is a possibility that bacteriochlorophyll is excited as a result of the transfer of excitation energy from re-excited magnesium protoporphyrin by inductive resonance, since the emission spectrum of luminescence from magnesium protoporphyrin overlaps the absorption spectrum of bacteriochlorophyll in the 590 nm region. However, in *Chromatium vinosum*, content of magnesium protoporphyrin and intensity of the delayed luminescence from it are negligible. Therefore, in the present paper we can analyze delayed fluorescence from bacteriochlorophyll without consideration of this possibility. In addition, delayed fluorescence from bacteriochlorophyll was studied in this work under aerobic conditions which were extremely unfavorable for the delayed fluorescence from magnesium protoporphyrin [14], and with infrared excitation light which was not absorbed by magnesium protoporphyrin.

## MATERIALS AND METHODS

*Chromatium vinosum*, strain D, was grown photoautotrophically in an inorganic medium described by Bose [15] at 27–30 °C. After 3–5 days, bacterial cells were harvested and chromatophores were prepared with a French pressure cell as described previously [16]. The preparation and suspending medium was 60 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose and 17 mM NaCl.

Reaction center-rich subchromatophore preparation, similar to the "heavy fraction" of Garcia et al. [17] or "fraction A" of Thornber [18], was obtained by an ammonium sulfate fractionation (20–30 g ammonium sulfate/100 ml water) after a treatment of chromatophores with sodium cholate (final concentration, 1 %). The spectral properties of the preparation obtained were similar to those reported by Garcia et al. [17] and Thornber [18].

Delayed fluorescence was measured with the phosphoroscope described previously [14, 19]. Actinic light was provided by a high-pressure mercury lamp in

combination with a Corning 9788 filter and chopped by a rotating sector. The intensity of the actinic beam at the sample position was about $10^5$ ergs · $cm^{-2}$ · $s^{-1}$. RCA 7102 photomultiplier covered with a Wratten 88A filter or a Corning 2600 filter was used to detect the emission from the sample. During the illumination period, the photomultiplier was covered by the rotating sector to be protected from the actinic beam and prompt fluorescence. Amplified signal was recorded on a storage oscilloscope or on a magnetic tape with a TEAC R-410 data recorder. For the measurement of the change of intensity during the repetitive illumination and for averaging decay traces, signals recorded on magnetic tapes were used. All the measurements were carried out at room temperature (15–25 °C).

The same phosphoroscopic instrument was used to measure absorption changes of samples with a single beam under the same conditions used for the measurement of delayed fluorescence [17]. Photooxidation of reaction center bacteriochlorophyll was measured by following the absorbance change at 789 nm. EMI 9558 photomultiplier covered with a Corning 2600 filter and a 789 nm-transmitting interference filter was used to detect the measuring beam. For measuring the absorption change, a sector chopped the actinic light in the same manner as for delayed fluorescence, but the sector did not cover the photomultiplier detecting the measuring beam.

A photon counter (NF Circuit Design Inc., Model PC-545A) was used for measuring emission spectra of the delayed fluorescence, since the emitted light was extremely weak after the spectral dispersion with a Shimadzu-Bausch and Lomb high intensity monochromator (with IR no. 1 grating). Spikes caused by photoelectrons were counted in a sampling period of 20 ms during the measuring time, and the count in the same length of sampling period during the illumination time (with the photomultiplier covered) was subtracted as the dark level. This process was repeated and data was accumulated for 500 cycles with an illumination period of 40 ms and a dark period of 40 ms in a cycle.

## RESULTS

In Fig. 1, the emission spectrum of delayed fluorescence from chromatophores is compared with that of prompt fluorescence measured with the same actinic light and in the same geometry of the apparatus. The accurate determination of the spectra was somewhat difficult owing to the rather wide measuring slit width and a low signal-to-noise level in the case of delayed fluorescence. With these limitations in mind, it may be said that general shapes of the two spectra were similar and positions of the peaks were close to each other (920–930 nm). This comparison shows that BChl* is the emitter of the observed delayed fluorescence.

Typical time courses of delayed fluorescence and oxidation of $P$ are shown in Fig. 2. Intensity of the delayed fluorescence was low just after the start of illumination and increased on successive intermittent illumination of 1–2 s. On the contrary, the profiles of oxidation-reduction of $P$ did not change during the intermittent illumination except the first one. The successive increase of delayed fluorescence is probably due to the accumulation of reduced acceptor, or formation of membrane potential.

Carithers and Parson [4] showed that at low redox potentials, where cytochromes were reduced and donated electrons rapidly to $P^+$, delayed fluorescence was not observed. Although we did not raise the redox potential to oxidize cytochromes

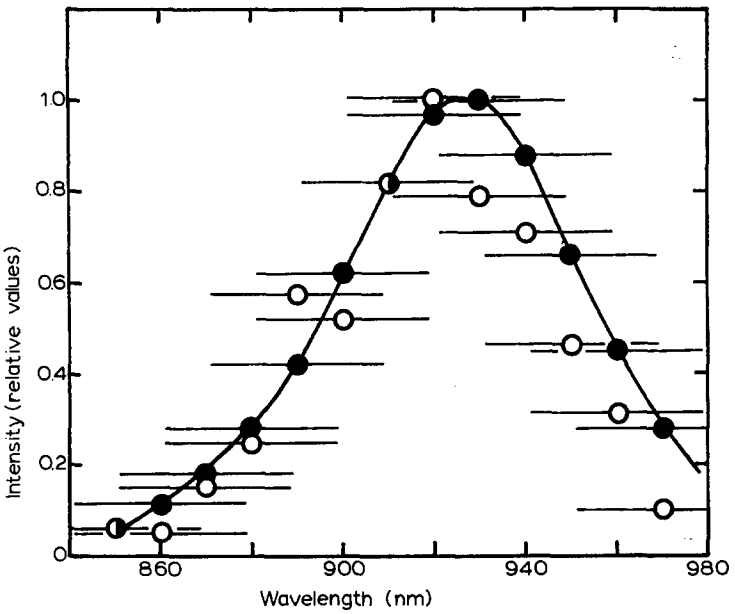



Fig. 1. Emission spectra of fluorescence from *Chromatium vinosum* chromatophores. ○, delayed fluorescence measured in the 5–25 ms after the cessation of illumination. Countings of photon signals were accumulated for 500 cycles, each with a 40 ms illumination period and a 40 ms dark period. ●, prompt fluorescence. Horizontal lines indicate the slit width of the monochromator. Intensities were normalized at the maximum value. Chromatophores were suspended in 60 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose and 17 mM NaCl. Concentration of bacteriochlorophyll, 6.5 $\mu$M (○) and 7.7 $\mu$M (●), respectively.

chemically, delayed fluorescence was observed. This difference is not essential because in our measurements cytochromes were photooxidized during the repetitive illumination. In preliminary experiments, oxidation of cytochrome(s) to a steady state level was achieved within 1 s.

In Fig. 3, decay time course of the delayed fluorescence in a single cycle of the repeated illuminations was compared with that of $P^+$ at pH 7.4 in semi-logarithmic plottings. Delayed fluorescence decayed biphasically. Half time of fast component was about 8 ms and that of slow one was about 100 ms. The half-reduction time of $P^+$ was similar to the half-decay time of the slow component of delayed fluorescence but not to that of the fast component.

Intensity and decay kinetics of the delayed fluorescence depended on the pH of the medium (Fig. 4). At higher pH, the slow phase was dominant and the decay of the slow component was accelerated. The intensity of the slow component increased at higher pH values. At low pH (pH 6 in Fig. 4), the fast component with a half time of about 8 ms could be clearly distinguished from the slower component with a half time of about 200 ms. As dark reduction of $P^+$ was slowed at low pH values (data not shown), the difference between the dark reduction of $P^+$ and the decay of the delayed fluorescence was much more distinctly observed at the beginning of the dark period (10–50 ms). Since the prompt fluorescence from bulk bacteriochlorophyll was not affected by the pH values tested, pH dependency of the intensity of delayed fluores-

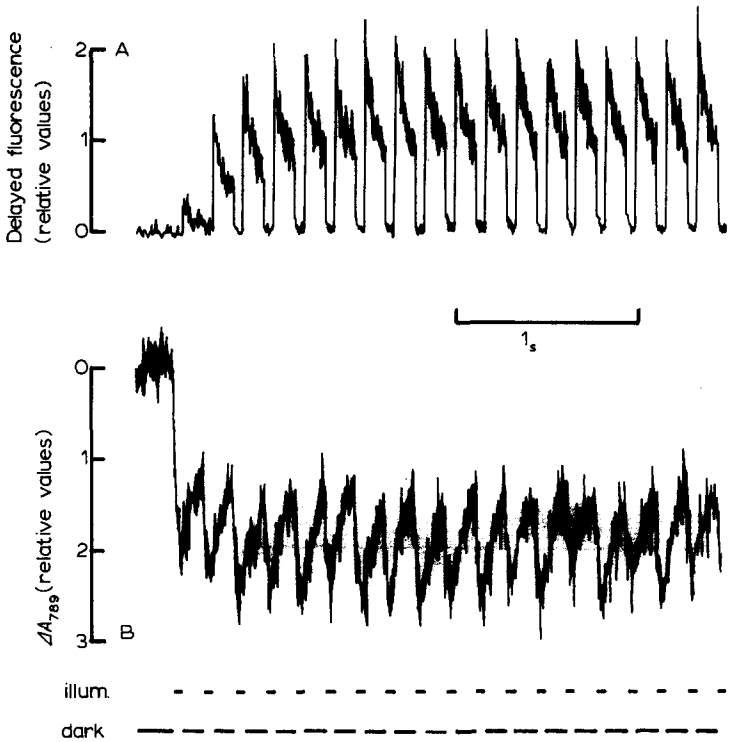



Fig. 2. Time courses of delayed fluorescence and oxidation of *P* during intermittent illumination. A, delayed fluorescence. B, absorbance change at 789 nm, the downward deflection corresponds to the oxidation of *P*. The bars at the bottom indicate the illumination and the dark periods; duration, 40 ms and 120 ms, respectively. Chromatophores were suspended in the same buffer as in Fig. 1. Concentration of bacteriochlorophyll, 29 $\mu$M.

cence was not a result of a difference of the fluorescence yields due to a change in the rate of nonradiative loss of excitation energy. The possibility cannot be ruled out that delayed fluorescence is emitted by a small fraction of fluorescing bacteriochlorophyll and, therefore, the fluorescence yield of the delayed-fluorescence-emitting molecules is not precisely reflected in the intensity of prompt fluorescence.

When electron transport between primary and secondary electron acceptor was inhibited in the presence of *o*-phenanthroline (3.3 mM), both the reduction of $P^+$ and the decay of delayed fluorescence in a single light-dark cycle were accelerated. In Fig. 5, the time courses of delayed fluorescence and reduction of $P^+$ in the presence of *o*-phenanthroline in a single cycle are compared. Reduction of $P^+$ was approximately first-order with a half time of about 15–20 ms. After the first 30 ms, the delayed fluorescence decayed with a time course similar to that of the reduction of $P^+$, while within 30 ms it decayed more rapidly than $P^+$. Prolonged illumination (longer than 20 s) decreased the intensity of the delayed fluorescence and the difference between the time course of delayed fluorescence and the reduction of $P^+$ became smaller. The oxidation level of $P^+$ and the time course of its re-reduction was not changed. CCCP caused effects similar to prolonged illumination in the presence of *o*-phenanthroline.

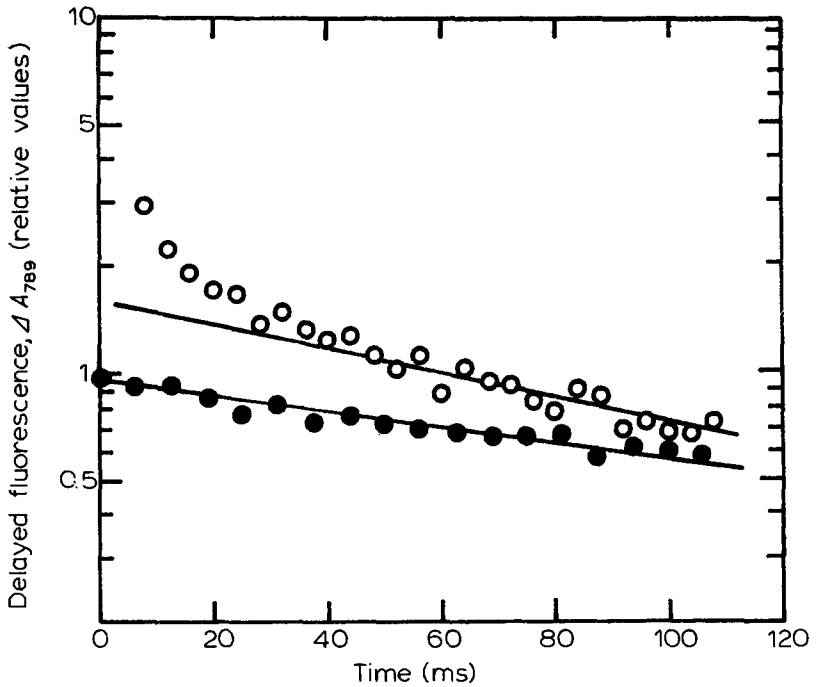



Fig. 3. Time courses of the decay of delayed fluorescence and reduction of $P^+$ in a single cycle. ○, delayed fluorescence; ●, $P^+$ measured from absorbance change at 789 nm. Illumination period, 40 ms; dark period, 120 ms. Measurements were performed after the intensity of the delayed fluorescence reached a steady state. Ten traces were averaged. Chromatophores were suspended in the same buffer as in Fig. 1. Concentration of bacteriochlorophyll, 29 $\mu$M.

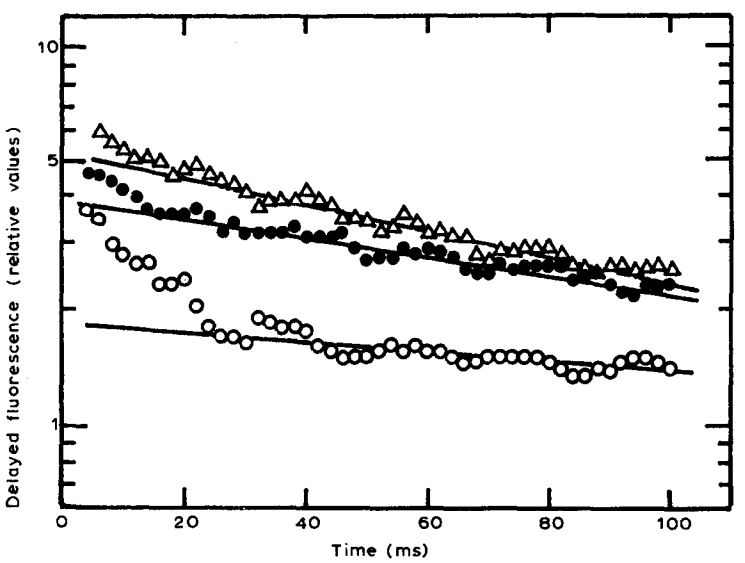



Fig. 4. Effect of pH on level and decay of delayed fluorescence. ○, pH 6; ●, pH 7; △, pH 8. Illumination period, 40 ms; dark period, 120 ms. 120 traces were averaged for respective pH. Chromatophores were suspended in 60 mM phosphate buffer containing 0.25 M sucrose and 17 mM NaCl. Concentration of bacteriochlorophyll, 20 $\mu$M.

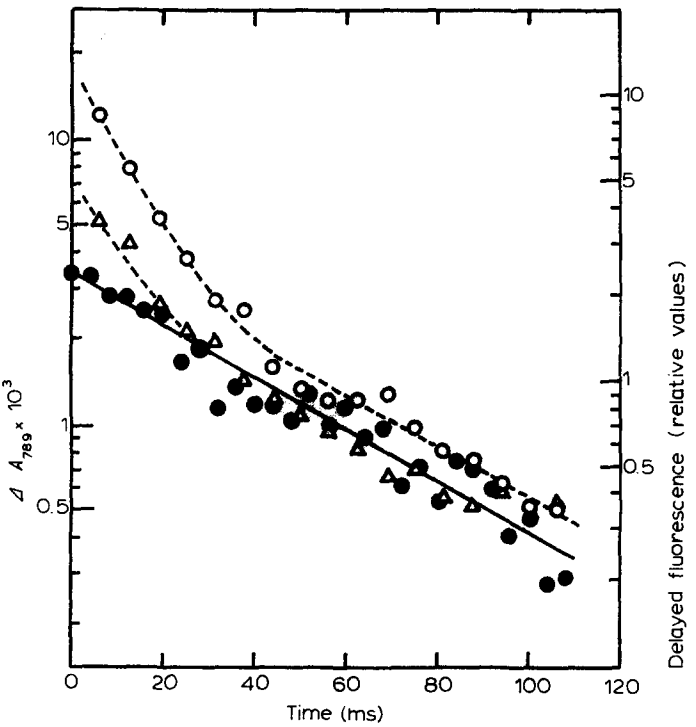



Fig. 5. Comparison of time courses of decay of delayed fluorescence and $P^+$ in a single cycle in the presence of *o*-phenanthroline. ○, delayed fluorescence at 1–2.4 s after the beginning of illumination; △, delayed fluorescence at 18.4–19.8 s; ●, $P^+$ measured from $\Delta A$ at 789 nm (calculated from photographic recordings taken during the period of 5–15 s from the start of illumination). Illumination period, 40 ms; dark period, 120 ms. 10 and 5 traces were averaged for delayed fluorescence and $P^+$, respectively. Chromatophores were suspended in the same buffer as in Fig. 1. Concentration of bacteriochlorophyll, 29 $\mu$M. *o*-Phenanthroline, 3.3 mM.

Delayed fluorescence from subchromatophore particles was extremely weak (lower than one-tenth of that of chromatophores on the basis of amount of $P^+$). Its decay time course coincided exactly with that of the reduction of $P^+$ (Fig. 6). Prolonged illumination and CCCP had no effect on either intensity and time courses of delayed fluorescence from the subchromatophore particles in the presence of *o*-phenanthroline.

Uncoupling agents such as CCCP or valinomycin plus nigericin decreased the slow component of delayed fluorescence very much. The decay of the slow phases was much accelerated (Figs. 7, 8). The dark reduction of $P^+$ was also accelerated by CCCP (Fig. 7). Nigericin accelerated the decay of the slow component of delayed fluorescence. Valinomycin decreased the intensity and did not accelerate the decay. In some cases the decay was made slower by valinomycin. The simultaneous presence of both valinomycin and nigericin further decreased the intensity and accelerated the decay than in the presence of either valinomycin or nigericin alone. The effects of valinomycin and nigericin were not observed in $K^+$-free chromatophore suspensions. Generally, reagents that dissipate the proton gradient (CCCP, nigericin) accelerated

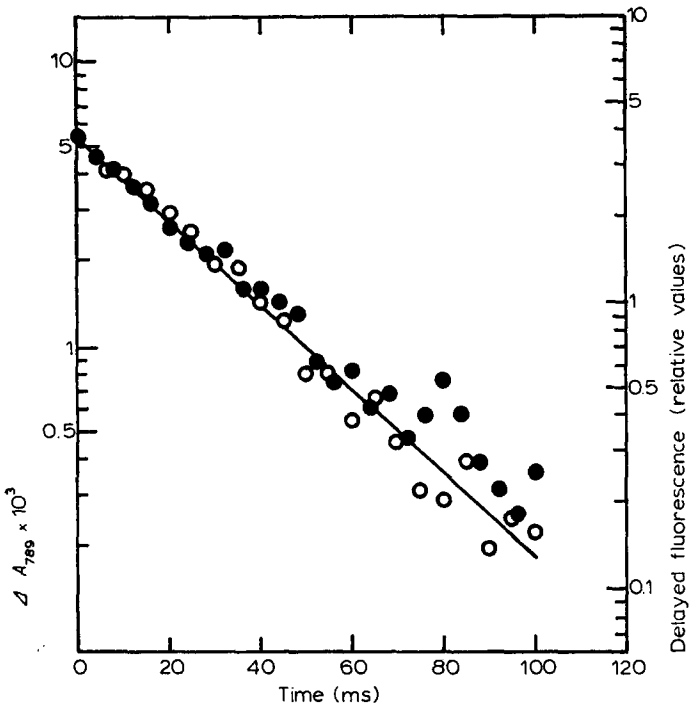



Fig. 6. Time courses of decay of delayed fluorescence and reduction of $P^+$ in subchromatophore preparation in the presence of *o*-phenanthroline. ○, delayed fluorescence; ●, $P^+$ measured from $\Delta A$ at 789 nm. Illumination period, 40 ms; dark period, 120 ms. 120 and 3 traces were averaged for delayed fluorescence and $P^+$, respectively. Subchromatophore preparations were diluted in the same buffer as in Fig. 1. Concentration of bacteriochlorophyll, 25 $\mu$M. *o*-Phenanthroline, 3.3 mM.

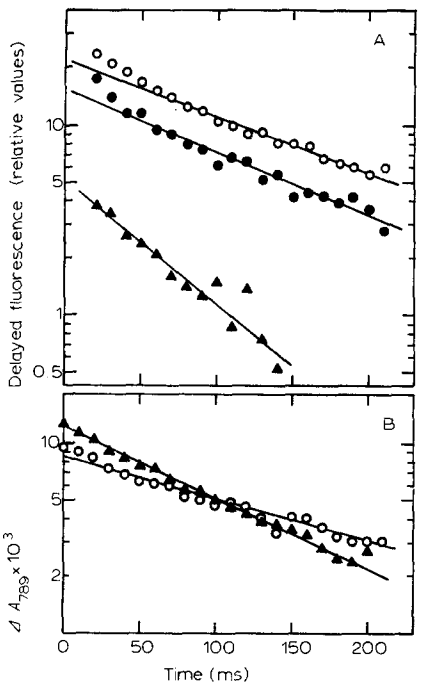
the decay, and reagents that dissipate the membrane potential (CCCP, valinomycin) decreased the intensity. The effects of membrane potential on the level of delayed fluorescence has been pointed out [8–12].

## DISCUSSION

### *Existence of two components in the decay time course of delayed fluorescence*

The intensity of the delayed fluorescence is determined by the number of singlet excited bacteriochlorophyll molecules, BChl*. The amount of BChl* formed after illumination is a function of the amount of $P^+A^-$ state, the activation energy for the process of recombination of $P^+$ and $A^-$, and other variables [6, 12]. Therefore, the decay of the delayed fluorescence reflects the disappearance of $P^+$ and $A^-$ formed by the light, or that is to say, "relaxation" of $P^+$ and $A^-$ until establishment of an equilibrium with the surrounding molecules in the microenvironment.

Apparently, there were two components (fast and slow) in the decay time courses of delayed fluorescence at acidic pH, although the fast component could not be clearly distinguished at higher pH values. The biphasic decay is interpreted as follows: since the dark reduction of $P^+$ is slower than the fast decay of delayed fluorescence, dark oxidation of $A^-$ but not the dark reduction of $P^+$ probably determines the



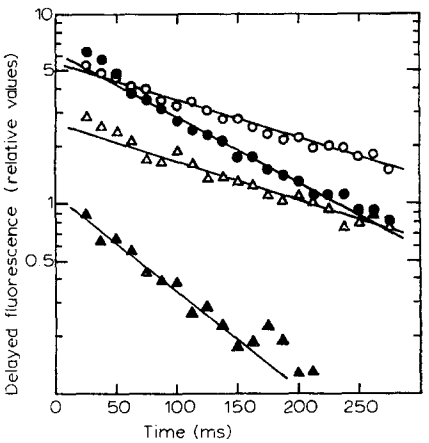




Fig. 7. Effect of CCCP on delayed fluorescence and reduction of $P^+$. A, delayed fluorescence. B, $P^+$ measured from absorbance change at 789 nm. ○, control; ●, $10^{-8}$ M CCCP; ▲, $10^{-7}$ M CCCP. Illumination period, 100 ms; dark period, 300 ms. Two traces were averaged. Chromatophores were suspended in the same buffer as in Fig. 1. Concentration of bacteriochlorophyll, 39 $\mu$M.

Fig. 8. Effects of valinomycin and nigericin on delayed fluorescence. ○, control; ●, $3.4 \cdot 10^{-8}$ M nigericin; △, $0.9 \cdot 10^{-8}$ M valinomycin; ▲, $3.4 \cdot 10^{-8}$ M nigericin and $0.9 \cdot 10^{-8}$ M valinomycin. Illumination period, 100 ms; dark period, 300 ms. Five traces were averaged. Chromatophores were suspended in 60 mM phosphate buffer composed of $NaH_2PO_4$ and $Na_2HPO_4$, pH 7.4, containing 100 mM KCl, 0.25 M sucrose and 17 mM NaCl. Concentration of bacteriochlorophyll, 21 $\mu$M.

fast decay of delayed fluorescence within 30 ms. If we assume that the electron transfer between A and the secondary acceptor is reversible and sufficiently rapid, $A^-$ would be oxidized until the equilibrium with the secondary acceptor is established (within 30 ms). The secondary acceptor, which becomes more reduced than the dark steady state during the short (but continuous) illumination, will reach an equilibrium with $A^-$ in the first 30 ms of the dark period (with a slight shift in the reduction level of the secondary acceptor pool, if the amount of the total secondary acceptor is larger than that of $P$). Thereafter, it will be oxidized slowly in the dark. In short, it may take about 30 ms for the quasi-equilibrium to be established between $A^-$ and the secondary electron acceptor. After that, the level of $P^+$ probably limits the level of delayed fluorescence; dark reduction of $P^+$ becoming the main determining factor of the slow decay of delayed fluorescence. Therefore, the pH dependence of the slow component decay probably reflects pH dependence of the dark reduction of $P^+$. However, the possibility is not ruled out that the intensity of the slow component is affected by a slow change of the reduction level of $A^-$, induced by slow oxidation of the reduced secondary electron acceptor.

*pH dependence of the decay and intensity of delayed fluorescence*

If the equilibrium between $A^-$ and the proton-binding secondary electron acceptor Q (probably quinone) is established, i.e.,

$$2A^- + 2H^+ + Q \rightleftharpoons 2A + QH_2,$$

then a decrease in $H^+$ is expected to cause a shift in the equilibrium with an increased amount of $A^-$. The increase of the slow component of delayed fluorescence at higher pH values may be due to the increase of $A^-$.

The apparent increase of the fast component at lower pH can be understood if we suppose that in the steady state of illumination the level of $A^-$ is not appreciably affected by pH. The difference between the level of $A^-$ at the time of cessation of illumination and the level after the establishment of an equilibrium with the secondary electron acceptor increases at lower pH, since the latter decreases at lower pH.

*Decay time course in the presence of o-phenanthroline*

Acceleration of the reduction of $P^+$ has been observed when electron transfer from the primary electron acceptor to the secondary acceptor was blocked [20, 21]. This phenomenon can be interpreted as the result of an accelerated return of electron from $A^-$ to $P^+$.

In this case, the number of the paired $P^+$ and $A^-$ is approximately the same as the number of $P^+$, because electrons trapped in $A^-$ cannot move to the secondary acceptor and their return to $P^+$ is the only process for oxidation of $A^-$. Fleischman [6] and Carithers and Parson [4] observed the identical time courses for the decay of delayed fluorescence and for the reduction of $P^+$ at low temperatures or in the presence of *o*-phenanthroline. In our study, the results with the subchromatophore particle were similar to their data. When "intact chromatophores" were used, the delayed fluorescence was somewhat intensified within the first 30 ms after the cessation of illumination. We think this phenomenon is probably a result of acceleration of BChl*-generating recombination reaction of $P^+$ and $A^-$ by electric field generated by systematically oriented $P^+$ and $A^-$ in intact chromatophore membranes. The field should be proportional to a number of the charges ($P^+$ and $A^-$), therefore, delayed fluorescence will be stimulated more distinctly at earlier stage than after the relaxation of most of the separated charges. Quantitative agreement with this interpretation has been obtained (paper in preparation).

*Effects of uncoupling reagents*

The changes in the intensity of delayed fluorescence in the presence of CCCP, valinomycin and nigericin correspond with the change in the electrical potential difference across the membrane expected from the well-known ion transporting characteristics of these reagents. For example, valinomycin increases the permeability of potassium ion and decreases the membrane potential. However, it did not completely quench the delayed fluorescence because the concentration gradient of potassium ion prevented the further movement of potassium ion. The simultaneous presence of valinomycin and nigericin completely dissipated the membrane potential and the delayed fluorescence was almost completely quenched.

CCCP and nigericin, which collapse the light-induced proton gradient and increase the internal pH of chromatophores, had the same effect as high pH on the

decay of the slow component of delayed fluorescence and $P^+$. It is probable that the internal pH determines the rate of electron transfer and, consequently, the decay of $P^+$ and delayed fluorescence. The effect of valinomycin on the decay of delayed fluorescence probably reflects the accumulation of protons to a higher level than in its absence, as a result of the dissipation of membrane potential.

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