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DELAYED FLUORESCENCE FROM *RHODOPSEUDOMONAS SPHAEROIDES* FOLLOWING SINGLE FLASHES

ROBERT P. CARITHERS* and WILLIAM W. PARSON

Department of Biochemistry, University of Washington, Seattle, Wash. 98195 (U.S.A.)

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SUMMARY

Delayed fluorescence from *Rhodopseudomonas sphaeroides* chromatophores was studied with the use of short flashes for excitation. Although the delayed fluorescence probably arises from a back-reaction between the oxidized reaction center bacteriochlorophyll complex (P^+) and the reduced electron acceptor (X^-), the decay of delayed fluorescence after a flash is much faster ($\tau_{\frac{1}{2}} \approx 120\ \mu s$) than the decay of P^+X^-. The rapid decay of delayed fluorescence is not due to the uptake of a proton from the solution, nor to a change in membrane potential. It correlates with small optical absorbance changes at 450 and 770 nm which could reflect a change in the state of X^-.

The intensity of the delayed fluorescence is 11–18-fold greater if the excitation flashes are spaced 2 s apart than it is if they are 30 s apart. The enhancement of delayed fluorescence at high flash repetition rates occurs only at redox potentials which are low enough ($<+240$ mV) so that electron donors are available to reduce P^+X^- to PX^- in part of the reaction center population. The enhancement decays between flashes as PX^- is reoxidized to PX, as measured by the recovery of photochemical activity. Evidently, the reduction of P^+X^- to PX^- leads to the storage of free energy that can be used on a subsequent flash to promote delayed fluorescence. The reduction of P^+X^- also is associated with a carotenoid spectral shift which decays as PX^- is reoxidized to PX. Although this suggests that the free energy which supports the delayed fluorescence might be stored as a membrane potential, the ionophore gramicidin D only partially inhibits the enhancement of delayed fluorescence. With widely separated flashes, gramicidin has no effect on delayed fluorescence.

At redox potentials low enough to keep X fully reduced, delayed fluorescence of the type described above does not occur, but one can detect weak luminescence which probably is due to phosphorescence of a protoporphyrin.

Abbreviations: P, P*, and P^+, the photosynthetic reaction center bacteriochlorophyll complex in its reduced form, its first excited singlet state, and its oxidized form, respectively; X, X^-, the primary electron acceptor in its oxidized and reduced forms, respectively; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; PMS, *N*-methylphenazonium methosulphate; *E*, oxidation-reduction potential relative to the standard hydrogen electrode; E_{mH}, mid-point redox potential of a symmetrical titration at the pH indicated in the subscript.

* Present address: Department of Cell Physiology, University of California, Berkeley, Calif. 94720, U.S.A.

INTRODUCTION

A previous paper [1] describes a study of delayed fluorescence from *Rhodopseudomonas viridis* chromatophores, following excitation with single flashes. The results support the view [1–3] that delayed fluorescence results from a reversal of the primary photochemical reaction of photosynthesis. In the photochemical reaction, an electron is driven from the reaction center bacteriochlorophyll complex (P_{870} or P) to an acceptor (X). The products of this reaction, the oxidized bacteriochlorophyll complex (P^+) and the reduced acceptor (X^-), ordinarily enter rapidly into secondary electron transfer reactions; but if the secondary processes are blocked, X^- can return an electron to P^+ in a back-reaction. In some cases, the back-reaction promotes P to an excited singlet state, P*. From P*, energy can migrate back to the bulk, antenna bacteriochlorophyll, and then be released as fluorescence.

Several investigators [3–6] have suggested that P^+ and X^- form an electric dipole perpendicular to the chromatophore membrane. If this is correct, the formation of P^+X^- must generate a transmembrane electrical potential, and the membrane potential could make an important contribution to the free energy that is needed to promote P^+ to P*. The inhibition of delayed fluorescence by ionophores has been explained in this way [3–5]. By increasing the conductivity of the membrane, ionophores would dissipate membrane potentials [7]. In our previous study [1], we observed only a relatively small inhibition of the delayed fluorescence by the potent ionophore gramicidin. We did not pursue this observation, because the *Rps. viridis* chromatophores seemed likely to be electrically leaky. For a meaningful study, one requires a preparation of tightly sealed membrane vesicles, and one would like to have an independent method for monitoring transmembrane potentials. Chromatophores isolated from *Rhodopseudomonas sphaeroides* are attractive in this regard, because when they are illuminated their endogenous carotenoids exhibit spectral shifts which have been attributed to transmembrane electrical fields [6]. In addition, much is known about the photochemical electron transfer reactions which occur in *Rps. sphaeroides* chromatophores [8, 9]. Although others have examined correlations between delayed fluorescence and carotenoid band shifts in *Rps. sphaeroides* [3, 10, 11] and *Rhodopseudomonas capsulata* [12], they have used phosphoroscopes which employ chopped, continuous light sources. Such instruments excite the sample repetitively at high rates and measure an average value of the delayed fluorescence. The use of flashes for excitation seemed likely to lead to a better understanding of how membrane potentials are related to the primary and secondary photochemical reactions. This paper describes such a study. The results are unexpectedly complex, and we have not been able to interpret all of them in a simple manner.

The chromatophore preparations that we have used in the present work were obtained by sonic oscillation of *Rps. sphaeroides* cells. This procedure detaches most of the endogenous cytochrome c_2 from the photochemical reaction center, greatly decreasing the rate at which the cytochrome can reduce P^+ [8, 9]. The increase in the lifetime of P^+ simplifies the technical problems of measuring delayed fluorescence after single flashes. One should bear in mind, however, that the detachment of the cytochrome renders the chromatophores somewhat unphysiological, and that this contributes to the complexity of the system. Dutton et al. [8, 9] have described the preparation of chromatophores in which cytochrome c_2 is largely still attached to the

reaction center. For similar technical reasons, most of our experiments have depended on the addition of an inhibitor of electron transfer, *o*-phenanthroline. This also complicates comparisons with studies [8, 9] of the uninhibited electron transfer system.

MATERIALS AND METHODS

Rps. sphaeroides strain 2.4.1 was grown as described previously [1] for *Rps. viridis*. Chromatophores were prepared by sonic oscillation as described previously [1], except that for most purposes the cells were broken in a solution containing 0.1 M potassium phosphate, pH 8.0, 5 mM $MgCl_2$ and a small amount of DNAase. For proton uptake experiments, the cells were broken in a solution containing 0.4 M sucrose, 1 mM Tris · HCl, pH 8.0, 60 mM KCl, 5 mM $MgCl_2$ and DNAase.

Delayed fluorescence measurements were made in a phosphoroscope that was similar to the one used previously [1]. A Xe flash lamp provided an excitation pulse with a width of about 20 μs. Light from the flash lamp was projected through a Schott BG-38 filter (unless otherwise noted), through a shutter window provided by a rapidly rotating blade, and then to the sample. Luminescence from the sample passed through a second shutter window provided by the same blade, through a Corning 2550 filter and then to a cooled RCA 7102 photomultiplier. For measurement of the delayed fluorescence emission spectrum, a monochromator was added before the photomultiplier and the 2550 filter was replaced with a Corning 3482 filter. For measurements in the visible region, the RCA 7102 photomultiplier was replaced with an uncooled EMI 9558 tube and a Corning 9782 filter was added to the BG-38 excitation filter.

The photomultiplier anode current passed to an operational amplifier producing a current-to-voltage transimpedance of 10^7 Ω for the RCA 7102 tube or $2 \cdot 10^6$ Ω for the EMI 9558 tube, and the amplified signal was captured with a Biomation model 802 transient recorder. Data from the transient recorder were averaged in a computer of average transients. Additional details on the construction of the phosphoroscope are available in ref. 13.

All luminescence measurements were made using a quartz cuvette which was equipped with Pt and calomel electrodes for monitoring the redox potential. For all measurements except those of proton uptake, the chromatophores were diluted in 0.1 M potassium phosphate buffer, pH 8.0, redox-mediating dyes were added and the sample was rendered anaerobic by bubbling with N_2. When specified, *o*-phenanthroline, gramicidin, and other inhibitors were added to the concentrated chromatophore suspensions before dilution.

Flash-induced absorbance changes were measured essentially as described previously [14], with the same Xe excitation lamp that was used for the luminescence measurements. The excitation flash was filtered by a Corning 2600 filter for visible absorbance measurements, and by Schott BG-38 and Corning 9782 filters for infrared measurements. A monochromator and complementary filters prevented excitation light from reaching the measuring photomultiplier. The absorbance measurements also employed repetitive excitation and signal averaging, with flash repetition rates comparable to those used in the measurements of delayed fluorescence (see figure legends). In both cases, the intensity of the excitation flashes was essentially saturating with respect to the photooxidation of P_{870}.

RESULTS

(1) *Dependence of delayed fluorescence on the flash repetition rate*

Fig. 1 shows typical measurements of the delayed fluorescence that follows excitation of *Rps. sphaeroides* chromatophores with short flashes under conditions which give the highest levels of luminescence. These conditions include (a) buffering the solution at a moderately low redox potential, (b) inhibiting the secondary electron transfer reaction with *o*-phenanthroline, and (c) exciting the sample with relatively short intervals between successive flashes. We shall consider each of these conditions in detail, beginning with the requirement for closely spaced flashes.

The upper four traces in Fig. 1 show measurements of the delayed fluorescence which results from the first, second, third and fourth flashes in a train of flashes spaced 2 s apart. The smoother trace at the bottom of the figure is an average of the luminescence over 100 flashes, beginning with the tenth flash. All of the data that are presented elsewhere in this report were obtained from averaged traces like this one. Note that the delayed fluorescence that follows the first flash is much weaker than that which follows later flashes. The luminescence is considerably stronger on the second flash, and by the third flash it has reached essentially its maximal level.

The amount that the delayed fluorescence increases in the course of a flash train depends on how closely together one spaces the flashes. Fig. 2 presents an analysis of this relationship. Panel A shows the final (maximal) intensity of the delayed fluorescence as a function of the time interval between the flashes, for control samples and for chromatophores treated with gramicidin D. These measurements were made at 21 °C; panel B shows similar measurements made at 5 °C. In all cases, luminescence increases from a low value for widely spaced flashes to a value that is many-fold higher for closely spaced flashes. We shall refer to the increase in the intensity of delayed fluorescence with increases in the flash repetition rate as "enhancement".

Examination of panels A and B of Fig. 2 reveals that the extent of the enhance-

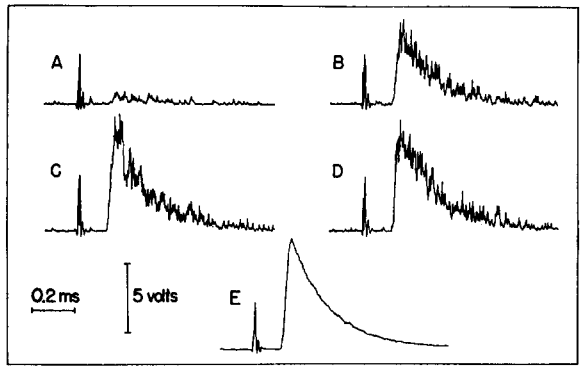

Fig. 1. Delayed fluorescence following the first (A), second (B), third (C), and fourth (D) flash in a series of flashes 2 s apart. An average of the 10th through the 110th flashes is shown in E. All traces are at the same amplification. Temperature, 20.5 °C; *E*, 207 mV; pH 8.0; *o*-phenanthroline, 1 mM; bacteriochlorophyll, 13.4 μM; DAD, 100 μM; PMS, 10 μM. The spike near the left in each trace indicates the time of the flash; the abrupt upward deflection to the right of this indicates the opening of the measuring shutter. The sample was allowed to relax in the dark for several minutes before the initiation of the flash series.

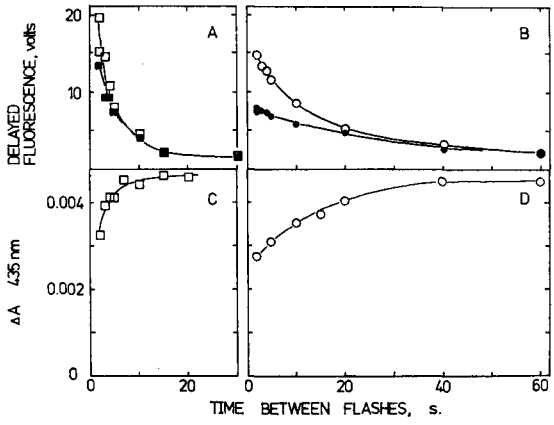

Fig. 2. (A) Intensity of delayed fluorescence, measured at 200 μs after flash excitation, as a function of the time between flashes. The delayed fluorescence in this and the following figures represents an average of many flashes after the luminescence has built up to its maximal value, usually commencing on the 5th or 6th flash (see text and Fig. 1). Open symbols, controls; filled symbols, plus gramicidin D (5 μg/ml). Bacteriochlorophyll, 11.7 μM; E, 225 mV; pH 8.0; *o*-phenanthroline, 1 mM; temperature 21 °C. B, same as A, except temperature 5 °C. C, Amount of P photooxidation as a function of the time between flashes. Flash-induced absorbance increases representing the formation of P^+ were measured at 435 nm. Bacteriochlorophyll, 31.4 μM; E, 207 mV; pH 8.0; *o*-phenanthroline, 1 mM; temperature, 21 °C; D, same as C, except temperature, 5 °C.

ment effect decreases approximately exponentially as a function of the time interval between the flashes and that the rate constant for the decay increases with temperature. The intensity of the luminescence following widely separated flashes is relatively insensitive to temperature, whereas the maximum extent of the enhancement resulting from closely spaced flashes increases with temperature. The effect of gramicidin is discussed below.

The enhancement of delayed fluorescence that results from spacing the flashes close together is not due to an increase in the extent of the primary photochemical reaction. Panels C and D of Fig. 2 show that the amount of P that is photooxidized on each flash actually decreases as the flashes are spaced closer together. The decrease of photochemistry probably is due to the accumulation of reaction centers in the photochemically inactive form PX^-; this will be discussed below.

(2) *Dependence of delayed fluorescence on electron transfer inhibitors*

As in *Rps. viridis* [1], delayed fluorescence from *Rps. sphaeroides* chromatophores increases when one adds an inhibitor of the secondary electron-transfer reaction in which ubiquinone [15] removes an electron from X^-. In the absence of such an inhibitor, the delayed fluorescence was unmeasureable. Fig. 3 shows the effect of adding *o*-phenanthroline [11, 15–17], the most effective agent that we tested in *Rps. sphaeroides*. The intensity of delayed fluorescence is half maximal at about 180 μM *o*-phenanthroline and maximal at about 1.5 mM. Although the measurements that were used for the figure were obtained with closely spaced flashes, *o*-phenanthroline had a similar effect on the weaker delayed fluorescence that followed widely spaced flashes.

Fig. 3 also shows parallel measurements of the extent to which *o*-phenanthro-

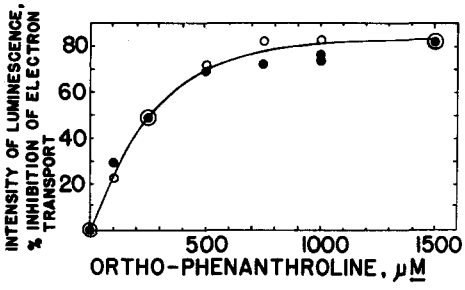

Fig. 3. Intensity of delayed fluorescence and inhibition of secondary electron transfer as a function of *o*-phenanthroline concentration. The percent inhibition of the electron transfer reaction (closed circles) is measured by the percentage of the flash-induced absorbance change at 870 nm which recovers by fast kinetics (see text). The open circles represent delayed fluorescence, measured at 200 μs after the flash. Bacteriochlorophyll, 13.4 μM; *E*, 375 mV (●) and 207 mV (○); 21 °C; pH 8.0; flash spacing 2 s (○) and 1 min (●); PMS, 10 μM; $K_4Fe(CN)_6$, 50 μM (●); DAD, 100 μM (○). The two curves are normalized at 1.5 mM *o*-phenanthroline, making the vertical scale arbitrary for the delayed fluorescence measurements.

line inhibits the secondary oxidation of X^-. These were obtained by examining the kinetics with which P^+ decayed at relatively high redox potentials, as shown in Fig. 4. At high potentials, cytochrome c_2 and other electron donors are largely oxidized, and in the absence of *o*-phenanthroline the reduction of P^+ is extremely slow (Fig. 4, trace A). If one prevents electrons from leaving X^- for ubiquinone, the back-reaction from X^- to P^+ can occur, reducing P^+ with a half-time of about 60 ms (Fig. 4, trace

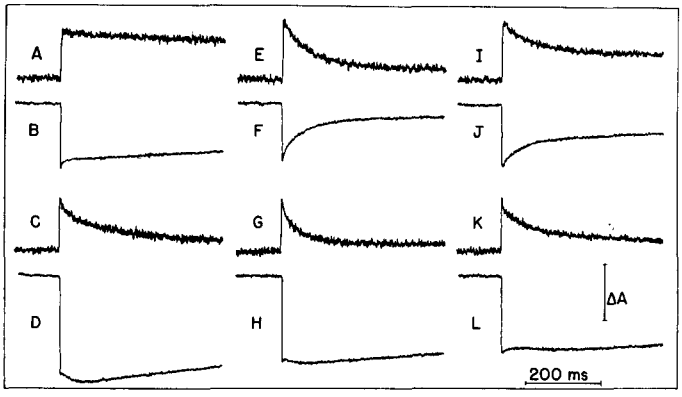

Fig. 4. Decay kinetics of P^+ and the carotenoid band shift. The traces exhibiting upward displacements (A, C, E, G, I and K) represent P photooxidation measured by the flash-induced absorbance decrease at 600 nm. The traces showing downward displacements (B, D, F, H, J and L) represent the carotenoid band shift as measured by the difference between the flash-induced absorbance changes at 523 and 508 nm. (The shift results in an absorbance increase at 523 and a decrease at 508 nm.) This experiment compares the recovery of P^+ and the carotenoid band shift at a relatively high redox potential (top two rows) and a moderately low potential (bottom two rows) in uninhibited chromatophores (first column), *o*-phenanthroline-inhibited samples (second column), and samples treated with piericidin A (third column). The vertical bar represents an absorbance change of 0.004 for P^+ and 0.010 for the carotenoid shift. 21 °C; 30 s spacing between flashes; pH 8.0; bacteriochlorophyll, 26.4 μM; redox potential, 395 mV (A, B, E, F, I, and J) and 205 mV (C, D, G, H, K, and L); 1 mM *o*-phenanthroline (E–H); 184 μM piericidin A (I–L).

E). The effectiveness of *o*-phenanthroline in blocking the forward reaction can be estimated by the fraction of the P^+ recovery which exhibits the rapid kinetics. By this criterion, saturating concentrations of *o*-phenanthroline inhibit the electron transfer reaction by about 83 % (Fig. 3). The data in Fig. 3 show that the effect of *o*-phenanthroline in increasing delayed fluorescence is directly proportional to its effect in inhibiting the oxidation of X^-. This is an important result for two reasons. First, it means that the enhancement of delayed fluorescence probably does not depend on effects [17, 18] of *o*-phenanthroline on steps other than the oxidation of X^-. Second, it indicates that electron flow through uninhibited reaction centers probably is not responsible for the enhancement effect. One might suppose that uninhibited reaction centers generate a species or condition that acts to enhance delayed fluorescence in inhibited centers. If this were the case, the basal level of delayed fluorescence would increase with increasing concentrations of *o*-phenanthroline but the enhancement would decline, contrary to our observations.

Although piericidin A [15] is also a moderately effective inhibitor of the secondary electron-transfer reaction (Fig. 4, trace I), it does not produce commensurately high levels of delayed fluorescence. With saturating levels of piericidin A (180 μM) the intensity of delayed fluorescence is only about 1 % of the intensity produced by saturating concentrations of *o*-phenanthroline. The small amount of luminescence that is elicited by piericidin is relatively insensitive to the flash repetition rate, i.e. it does not exhibit enhancement. Further, piericidin A antagonizes the effect of *o*-phenanthroline. If chromatophores which contain 1 mM *o*-phenanthroline are supplemented with 90 μM piericidin A, the intensity of the delayed fluorescence decreases, and the decrease is more pronounced at high flash repetition rates than it is at lower ones. The intensity decreases by 70 % with a 2-s spacing between flashes and by 40 % with an 11 s spacing.

Several other agents which block the secondary electron transfer reaction in *Chromatium vinosum* [15] and *Rps. viridis* [1] are ineffective inhibitors in *Rps. sphaeroides*. These include 5-*n*-pentadecyl-6-hydroxy-4,7-dioxobenzothiazole, 7-*n*-pentadecyl-6-hydroxy-5,8-quinolinequinone, 6-*n*-dodecylamino-5,8-quinolinequinone, 6-ω-cyclohexyloctyl-7-hydroxy-5,8-quinolinequinone, 6-cycloheptylamino-2-methyl-5,8-quinolinequinone, 5-*n*-octadecylmercapto-2,3-dimethyl-1,4-benzoquinone, 2-*n*-tetradecylamino-1,4-naphthoquinone, and 7-*n*-tetradecylmercapto-6-hydroxy-5,8-quinolinequinone. These compounds elicit delayed fluorescence in *Rps. viridis*, as does piericidin A [1].

Antimycin A, which blocks electron transfer between cytochromes *b* and c_2 [8, 9], inhibits *o*-phenanthroline-induced delayed fluorescence by about 14 % at 4.6 μM in *Rps. sphaeroides*. This does not appear to depend on the flash repetition rate. The insensitivity of the delayed fluorescence to antimycin probably reflects the fact that the addition of *o*-phenanthroline and the detachment of cytochrome c_2 from the reaction center inhibits other electron transfer reactions severely, so that the reaction between cytochromes *b* and c_2 is not rate limiting.

(3) *Dependence of delayed fluorescence on the redox potential*

In *Rps. viridis*, delayed fluorescence is maximal at redox potentials near +420 mV [1]. At higher potentials, photochemical activity declines because P is oxidized in the dark before the flash. At lower potentials, membrane-bound *c*-type cytochromes

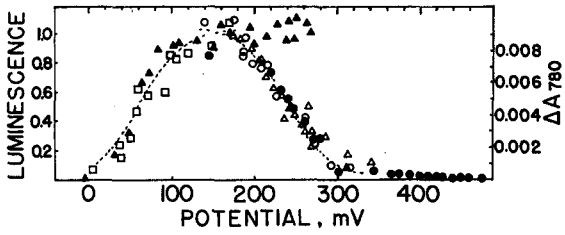

Fig. 5. Potentiometric titrations of delayed fluorescence and photochemical activity. Filled triangles represent P photooxidation measured from the flash-induced absorbance increase at 780 nm. All other symbols represent delayed fluorescence 200 μs after the flash. Flash spacing, 2 s for all measurements; 1 mM *o*-phenanthroline; pH 8.0; 21 °C; ▲ (photochemical activity), bacteriochlorophyll, 26.8 μM; redox buffers: 2,6-dichlorophenolindophenol, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, DAD, methylene blue, PMS, anthraquinone-2-sulfonate, and indigodisulfonate (all 10 μM). □ (delayed fluorescence), bacteriochlorophyll, 31.1 μM; redox buffers as for ▲. ● (delayed fluorescence), bacteriochlorophyll, 31.1 μM; redox buffers: 2,6-dichlorophenolindophenol, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, DAD, PMS, $K_4Fe(CN)_6$ (all 10 μM). ○ (delayed fluorescence), bacteriochlorophyll, 31.1 μM; redox buffers: 2,6-dichlorophenylindophenol (100 μM), $K_4Fe(CN)_6$ (10 μM), methylene blue (10 μM). △ (delayed fluorescence), bacteriochlorophyll, 31.4 μM; redox buffers: PMS (10 μM), *p*-methylaminophenol sulfate (100 μM). The vertical scale for the delayed fluorescence measurements is arbitrary.

are reduced, and can prevent delayed fluorescence by reducing P^+ quickly after the flash. As pointed out above, the *c*-type cytochrome (c_2) of *Rps. sphaeroides* is a soluble protein and is largely dissociated from the membrane when one prepares chromatophores by sonic oscillation, as we have done. To the extent that this occurs, the quenching of P^+ by the cytochrome should be relatively slow. One would expect, therefore, that a redox titration of delayed fluorescence in *Rps. sphaeroides* chromatophores would reveal a very broad maximum. At high potentials, delayed fluorescence should decrease with the +440 mV E_m of P [8], but at low potentials it might remain strong until one approaches the −20 mV E_m of X. (This is the apparent E_m of X at pH 8.0 in the presence of *o*-phenanthroline [18].) The experimental results are quite different, as Fig. 5 shows. Delayed fluorescence is maximal at a potential between +120 and +200 mV. The intensity of luminescence decreases on the low-potential side with an apparent E_m near +60 mV and it decreases on the high-potential side with an apparent E_m of about +240 mV. At low redox potentials, the intensity of the delayed fluorescence does not actually go to zero. Instead, one observes a very weak luminescence which differs in several respects from the delayed fluorescence that occurs at higher potentials. This will be discussed in a later section.

The decline of delayed fluorescence in the +60 mV potential region appears to result mainly from a decrease in the amount of photooxidation of P that occurs on each flash. This is shown by the solid triangles in Fig. 5. We attribute the loss in photochemical activity to an extensive accumulation of reaction centers in the inactive state PX^-. This state would arise if P^+ accepts an electron from cytochrome c_2, or from any reductant other than X^-. In the presence of *o*-phenanthroline, the oxidation of X^- would be slow and the photochemically inactive form of the reaction center would accumulate. This could happen at potentials well above the E_m of X.

Some conversion of the reaction centers into the state PX^- must also occur at the redox potentials between +120 and +200 mV at which delayed fluorescence is

maximal. One can see this by examining the rates at which P^+ is reduced after flashes in the presence and absence of *o*-phenanthroline (Fig. 4). In the absence of the inhibitor, the reduction of P^+ is considerably faster at a potential of +205 mV than it is at +395 mV (Figs. 4A and 4C). The faster rate at +205 mV presumably reflects the transfer of electrons to P^+ from donors other than X^-. If one adds *o*-phenanthroline to the sample at +205 mV, the reduction of P^+ is faster still, presumably because the back-reaction and the reduction by other electron donors now occur in parallel (Fig. 4G).

Comparison of Figs. 4C, 4E, and 4G indicates that approximately half of the reaction centers are converted into the photochemically inactive state PX^- after flash excitation at redox potentials near +200 mV. We therefore interpret the increase in photochemical activity that occurs when one increases the spacing between the flashes (Figs. 2C and 2D) as reflecting the rate of reoxidation of state PX^-. Comparison of the curves in Figs. 2A and 2B with those in Figs. 2C and 2D shows that the reoxidation of PX^- appears to parallel the decay of the enhancement effect.

As one lowers the potential through the +60 mV region, the reoxidation of PX^- evidently becomes extremely slow, so that photochemical activity declines severely (Fig. 5). Although the experiments of Fig. 5 were performed with closely spaced flashes, a similar decrease in delayed fluorescence occurred at low potentials with flashes that were spaced 30 s apart. It seems likely that the reoxidation of PX^- which occurs on the time scale of 2–60 s at higher redox potentials (Figs. 2C and 2D) requires the participation of an additional electron carrier, and that this pathway can be closed by reduction of the carrier. Among the carriers that might play such a role are ubiquinone, *b*-type cytochromes, and the redox buffers which were added as potentiometric mediators in the titrations.

The decrease in delayed fluorescence as one increases the potential above +200 mV (Fig. 5) appears to represent a titration of the enhancement effect, rather than a decrease in photochemical activity. Whereas increasing the flash repetition rate from 0.033 to 0.5 s^{-1} increased the intensity of delayed fluorescence by a factor of about 18 at +205 mV, the same change in repetition rate at +375 mV caused the luminescence to decrease by about 37 %. (The increase in the repetition rate caused the amount of photooxidation of P to decrease by about 30 % at both redox potentials. At the higher potential, the decrease in photochemical activity could be due to an accumulation of reaction centers in the inactive state P^+X, rather than the form PX^-. This could occur if the *o*-phenanthroline block is imperfect and if the redox potential is high enough so that the reduction of P^+ between flashes becomes rate limiting.)

The development of the enhancement effect as one lowers the potential between +300 and +200 mV (Fig. 5) parallels the reduction of electron donors which can compete with X^- in transferring electrons to P^+ (Fig. 4). Evidently, the enhancement of delayed fluorescence is associated with conditions that favor the reduction of P^+X^- to PX^- after a flash. This is consistent with the observation that enhancement and a decrease in photochemical activity develop in parallel when the flash repetition rate is increased (Fig. 2).

(4) *Correlations between delayed fluorescence and carotenoid band shifts*

Fig. 4 includes measurements of the carotenoid band shift that results from flash excitation under various conditions, and Fig. 6 shows additional measurements

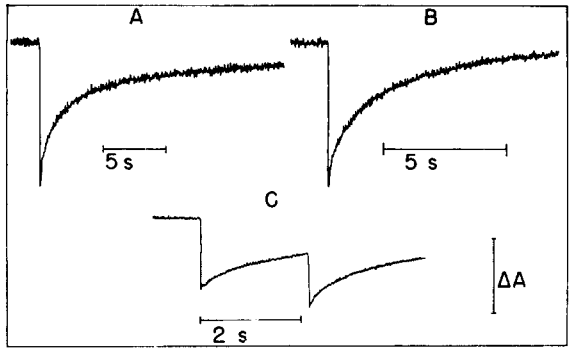

Fig. 6. (A) Carotenoid band shift resulting from a single flash at 4.5 °C. The downward deflection represents an increase in the absorbance at 523 nm. (B) Same as A, but at 21 °C. (C) Carotenoid band shifts on each of two flashes spaced 2 s apart at 21 °C. The vertical bar represents an absorbance change of 0.004 for A and B and 0.010 for C. Bacteriochlorophyll, 31.4 μM; *o*-phenanthroline, 1 mM; *E*, 208 mV; PMS, 10 μM; DAD, 100 μM. The flash groups were widely separated.

on a longer time scale. The upper six traces in Fig. 4 show that, at high redox potentials, the decay of the carotenoid band shift (traces B, F and J) parallels the decay of the corresponding P^+ signal (traces A, E and I). Whereas the rate of P^+ reduction increases at lower redox potentials (traces C, G and K), the decay of the carotenoid band shift becomes slower and a major part of the band shift persists after the complete decay of P^+ (traces D, H and L). Under conditions which allow donors other than X^- to reduce P^+, the amplitude of the band shift actually increases during the first 100 ms after the flash. Increases in carotenoid and bacteriochlorophyll band shifts accompanying reduction of P^+ by cytochromes have been observed previously in *Rps. sphaeroides* [19] and *C. vinosum* [20]. *o*-Phenanthroline decreases the extent of the secondary increase in the carotenoid shift after the flash (compare traces D and H), presumably because some of the electrons reducing P^+ now come from X^-, and this reaction collapses the carotenoid band shift in those units in which it occurs (Fig. 4, trace F). The carotenoid band shift which does build up after the flash in samples treated with *o*-phenanthroline decays quite slowly (Fig. 6). The decay of the carotenoid band shift appears to parallel the decay of enhancement, as measured by the curves in Figs. 2A and 2B, or the decay of PX^- to PX, as measured in Figs. 2C and 2D.

Because of its slow decay, the carotenoid band shift can build up in a cumulative manner during a flash train. This is illustrated by the lower trace in Fig. 6, which shows the response of the carotenoids to two flashes spaced 2 s apart. Immediately following the second flash, the cumulative amplitude of the band shift is about 30 % greater than it is after the first flash. Under the same conditions, the delayed fluorescence has increased about 9-fold (Fig. 1).

(5) *Effects of gramicidin*

In the presence of 5 μg/ml gramicidin D, the carotenoid band shift is undetectable by 50 μs after the flash. This is the case both with high flash repetition rates (0.5 s^{-1}) and with widely spaced flashes. Fig. 2 (panels A and B) shows, however, that gramicidin D has no significant effect on the delayed fluorescence that results from

widely spaced flashes. On the other hand, gramicidin does decrease the extent of enhancement. The decrease in the enhancement is temperature dependent, being more pronounced at lower temperatures. If the flashes are spaced 2 s apart, gramicidin decreases the delayed fluorescence by about 50 % at 5 °C (Fig. 2B) and by about 23 % at 21 °C (Fig. 2A). Even under these conditions, however, the enhancement effect is still quite dramatic. Enhancement evidently does not depend primarily on the build-up of a transmembrane electrical potential.

(6) *Decay kinetics of delayed fluorescence; proton uptake; fast absorbance changes*

Fig. 4 shows that, under conditions that give maximal delayed fluorescence, the major part of the population of P^+ decays with half-time of about 16 ms. This decay represents reduction of P^+ by electrons from both X^- and other electron donors. Because the oxidation of X^- by ubiquinone is blocked by *o*-phenanthroline, the kinetics with which P^+ decays should provide a measure of the rate of decay of P^+X^-. The delayed fluorescence, however, decays much more rapidly than this. At room temperature the half-time of its decay is about 120 μs (Fig. 1). The rate of the luminescence decay decreases somewhat with decreasing temperature: at 33.0, 20.0, 15.8, 11.0, 5.3 and 1.5 °C, the half-times were 103, 129, 143, 144, 152, and 155 μs, respectively, giving an Arrhenius activation energy of about 2.3 kcal/mol. The value of the activation energy is independent of the flash repetition rate; enhancement affects only the intensity of luminescence, not its decay rate.

This enormous discrepancy between the decay rates of P^+X^- and delayed fluorescence was not observed in *Rps. viridis* [1]. To test the possibility that our culture of *Rps. sphaeroides* was contaminated with a strain of bacteria which was highly luminescent but contributed little to the total P^+ signal, six individual clones were isolated from the culture. All of them yielded chromatophores with the same fast decay of luminescence. It appears, therefore, that some process must act rapidly after the flash to decrease the probability that P^+X^- will enter into the back-reaction that regenerates P*. This must be distinct from the process which causes the enhancement effect. The rapid quenching of the delayed fluorescence cannot be attributed to changes in transmembrane electrical potentials, because gramicidin has very little effect on the decay kinetics. (The decay was about 5 % slower in the presence of gramicidin.)

Among the processes which could occur to change the probability of luminescence after the flash is the uptake of a proton. Potentiometric titrations have shown that reduction of X to X^- is coupled to the binding of one proton, although this process probably is not rapid enough to occur during the lifetime of X^- under normal conditions [18, 21–23]. Proton uptake from the solution occurs following flash excitation, but this appears to be associated with the reduction of ubiquinone in the secondary electron transfer reaction. In the presence of *o*-phenanthroline, however, protonation of X^- might be rapid enough to decrease the free energy of P^+X^- substantially [22]. To consider this possibility, we measured proton uptake from the solution, following flash excitation under various conditions. In the absence of *o*-phenanthroline, the proton uptake had a half-time of about 360 μs (Fig. 7B), agreeing with measurements reported by Petty and Dutton [21]. This value is three times greater than the 120 μs half-time for the decay of delayed fluorescence (measured in the presence of *o*-phenanthroline). The addition of 1 mM *o*-phenanthroline decreased the amplitude of the proton uptake by about 70 % without significantly altering the

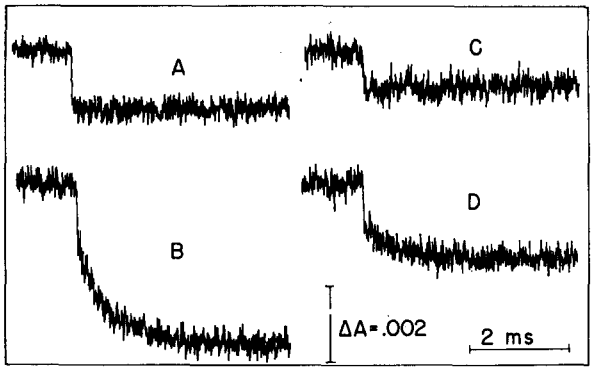

Fig. 7. Proton uptake following flash excitation. Experiments on the left show flash-induced absorbance changes in uninhibited chromatophores; those on the right, comparable measurements in samples inhibited with *o*-phenanthroline. The upper traces (A, C) were measured in 0.1 M potassium phosphate, pH 8.0, and represent absorbance changes due almost entirely to formation of P^+. The lower traces (B, D) were measured in 0.1 M KCl, pH 8.0, and exhibit absorbance changes due to the pH indicator (phenol red) as well as those associated with P^+. Bacteriochlorophyll, 18.4 μM; *o*-phenanthroline, 1 mM (C, D) and none (A, B); phenol red, 50 μM; measuring wavelength, 565 nm; flash spacing, 2 s; *E*, 205 mV; 21 °C.

kinetics of the remaining portion (Fig. 7D). Cogdell et al. [23] have reported observing an inhibition of 30–50 % by 4 mM *o*-phenanthroline, depending on the redox potential. A 70 % inhibition of proton uptake is only slightly less than the inhibition of about 80 % which *o*-phenanthroline exerts on the secondary electron transfer reaction (Fig. 3). Interpreted most simply, these results would indicate that most of the photosynthetic units in which the electron transfer is inhibited do not take up protons from the solution on the time scale of interest. Because these are the units which luminesce proton uptake would not appear to be responsible for the rapid decay of delayed fluorescence. An additional argument against the involvement of proton uptake in the rapid decay of delayed fluorescence is that the decay kinetics of the delayed fluorescence are independent of pH between pH 5 and 10, whereas the half-time of proton uptake increases with the pH (in the absence of *o*-phenanthroline) [21].

One should bear in mind that the experiments of Fig. 7 consider only the uptake of protons from the solution, and probably only from the solution on the outside of the chromatophore membrane. They might not reveal the rapid transfer of a proton to X^- from a component that equilibrates only slowly with buffers in the solution. Prince and Dutton [22] have suggested that *o*-phenanthroline itself can donate a proton to X^-, and that the resulting stabilization of X^- as XH accounts for the inhibition of the secondary electron transfer reaction. This suggestion would be consistent with our observations, if one assumes that the reprotonation of the *o*-phenanthroline by the solution is slow, or that it involves a region of the solution that is inaccessible to the pH indicator that we used. On the other hand, it would not explain the marked difference between the decay kinetics of delayed fluorescence in *Rps. viridis* and *Rps. sphaeroides*. The effects of *o*-phenanthroline on electron transfer and proton uptake are similar in the two species [1].

We next sought optical absorbance changes that might reveal other events occurring in the first half ms after the formation of P^+X^-. Fig. 8 shows typical mea-

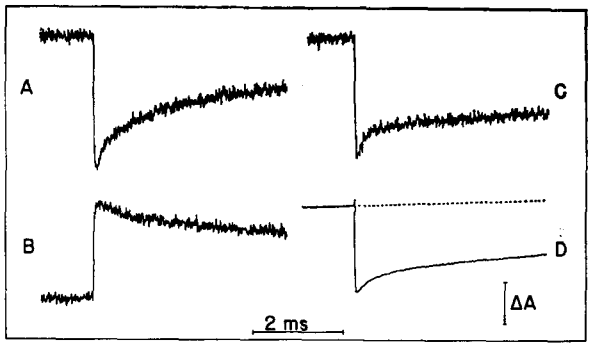

Fig. 8. Flash-induced absorbance changes at various wavelengths. Traces A, B, C, and D show absorbance changes at 420, 600, 450 and 770 nm, respectively. The vertical bar (ΔA) represents an absorbance change of 0.001 for traces A, B, and C and a change of 0.0028 for trace D. In D, the infrared photomultiplier tube gives a slowly rising baseline (broken line). Bacteriochlorophyll, 26.8 μM; *o*-phenanthroline, 1 mM; *E*, 205 mV; 21 °C; gramicidin, 5 μg/ml; flash spacing, 2 s.

surements at several different wavelengths. These were made in the presence of gramicidin, so as to avoid interference from carotenoid shifts. At 600 nm, the absorbance decrease which follows the flash reflects the photooxidation of P to P^+. On the time scale of this figure, the subsequent reduction of P^+ is relatively slow. The initial absorbance increases at 420, 450, and 770 nm also are due mainly to the formation of P^+, but at these wavelengths the initial changes are followed by rapid, partial reversals. Much of the reversal at 420 nm probably is due to the oxidation of cytochrome c_2; similar absorbance changes occur at wavelengths around 550 nm, coinciding with the α-band of the cytochrome. The kinetics of the fast phase of the recovery at 450 and 770 nm are faster than the predominant part of the recovery kinetics at 420 nm, and are similar to the decay kinetics of delayed fluorescence. Secondary absorbance changes of this type were seen only at moderately low redox potentials (a titration gave an E_{m8} of approx. +240 mV), but they were not affected by *o*-phenanthroline or the flash repetition rate (see ref. 13 for details). Because of the interference from the much larger absorbance changes due to P^+ and cytochromes, it was difficult to extract an accurate spectrum of the absorbance changes which parallel the decay of luminescence. The major features of the spectrum, however, appear to be peaks near 450 and 770 nm. In this regard, the absorbance changes are reminiscent of those which Clayton and Straley [24] and Slooten [25] have associated with changes in the redox state of X.

(7) *Spectral analysis of delayed fluorescence; luminescence at very low potentials*

The delayed fluorescence that is described above differs in several ways from what one might have expected to find. Its decay is anomalously fast, and its amplitude varies unconventionally with the redox potential and the flash repetition rate. These unusual properties raised the possibility that the delayed fluorescence might originate in a pigment which is not an integral part of the photosynthetic apparatus. This concern was heightened by the report of Arata et al. [26] describing phosphorescence from protoporphyrin IX in *Rps. sphaeroides* cells and chromatophores. We therefore investigated the emission spectrum of the delayed fluorescence.

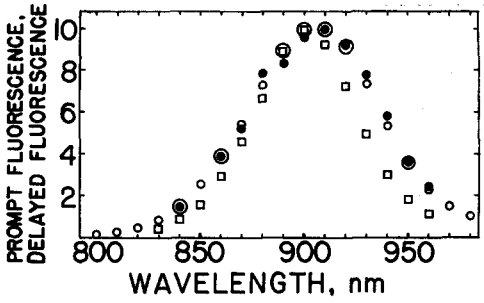

Fig. 9. Near infrared emission spectrum of *Rps. sphaeroides* fluorescence. Delayed fluorescence (●) at 200 μs after the flash was measured with a monochromator bandpass of about 50 nm. Prompt fluorescence was measured with the same bandpass (○) and with a 16-nm bandpass (□). Corrections were made for photomultiplier and monochromator sensitivity using a 2700 °K black-body radiation source. Bacteriochlorophyll, 23.4 μM (●) and 31.4 μM (○, □); *o*-phenanthroline, 1 mM; pH 8.0; temperature, 8 °C (●) and 20 °C (○, □); E, uncontrolled but with 10 μM DAD, mostly reduced; prompt fluorescence excited at 523 nm measured as described previously [1]. The vertical scales are arbitrary and are normalized at 900–910 nm,

Because of the weakness of the delayed fluorescence, measurements of the emission spectrum must involve relatively low optical resolution. The errors that this introduces can be recognized by comparing the emission spectra of prompt and delayed fluorescence. Fig. 9 shows that the emission spectrum of the delayed fluorescence is the same as that of prompt fluorescence, when the two are measured with similar resolution. The similarity of the two spectra indicates that the delayed fluorescence is emitted by the bulk light-harvesting bacteriochlorophyll. Increasing the resolution sharpens the spectrum of the prompt fluorescence emission, and indicates that the true maximum is near 900 nm.

An extremely weak delayed fluorescence in the near infrared can be detected even at potentials below −200 mV, when X is fully reduced. Because the photooxidation of P is prevented under these conditions, the luminescence cannot result from a back reaction between P^+ and X^-. The emission spectrum of the luminescence that occurs at very low potentials has major peaks near 600 and 650 nm, in addition to the peak near 900 nm (see ref. 13 for details). Whereas the luminescence at higher potentials has an excitation spectrum which appears to parallel the absorption spectrum of the bulk pigments, the low-potential luminescence has an excitation spectrum which is dominated by a sharp peak near 420 nm [13]. From these features, we attribute the luminescence at low potentials to the protoporphyrin IX phosphorescence described by Arata et al. [26]. The emission that occurs in the infrared under these conditions is presumably prompt fluorescence of the bacteriochlorophyll which is excited by 600 and 650 nm phosphorescence of the protoporphyrin. The intensity of the delayed fluorescence at low potentials is unaffected by the presence or absence of *o*-phenanthroline, or by the flash repetition rate. The decay half-time is about 310 μs, and neither this nor the intensity of the luminescence changes with temperature between 0 and 35 °C. The protoporphyrin phosphorescence probably does not make any significant contribution to the near infrared emission at higher potentials, because the luminescence that occurs at higher potentials is completely dependent on the presence of *o*-phenanthroline (Fig. 3).

DISCUSSION

Study of *Rps. viridis* chromatophores has linked delayed fluorescence to a reversal of the primary photochemical reaction [1, 2]. The present work is consistent with this picture, but it also emphasizes the point that secondary processes can modulate the intensity of delayed fluorescence enormously. In *Rps. sphaeroides*, delayed fluorescence decays after a flash much more rapidly than P^+X^- does. The decay evidently is not due to the uptake of a proton from the solution, but it is associated with small optical absorbance changes which could reflect a change in the state of X^-. Additional work will be necessary to clarify the meaning of these observations.

Another secondary process also modulates the intensity of delayed fluorescence strongly in *Rps. sphaeroides*, and it is mainly this effect which gives the delayed fluorescence its sensitivity to temperature, redox potential, ionophores, and the flash repetition rate. The conditions which lead to enhancement of delayed fluorescence, repetitive excitation at moderately low redox potentials in the presence of *o*-phenanthroline, are associated with two other phenomena. First, part of the reaction center population is reduced from state P^+X^- to PX^- following each flash. The return of these reaction centers to their photochemically active state (PX) is relatively slow, and appears to match the decay of the enhancement effect kinetically (Fig. 2). Second, the reduction of P^+X^- is accompanied by a carotenoid band shift which also decays with approximately the same kinetics. Although the three phenomena are not necessarily related causally, it would appear that the reduction of P^+X^- to PX^- (or some process which rapidly follows this step) leads to the storage of free energy that can be used after a subsequent flash to promote P^+X^- to the excited state P*X.

The enhancement of delayed fluorescence is most pronounced if the second flash follows closely after the first, at a time when reaction centers that have been converted to state PX^- appear not to have recovered photochemical activity. This would suggest that the free energy which is captured in the reduction of P^+X^- to PX^- is not used in the same reaction center where the reduction occurs, but rather is made available to other reaction centers which are photochemically active at the time of the second flash.

One interpretation of these observations would be that the reduction of P^+X^- (or a subsequent step) generates a transmembrane electrical potential. The carotenoid band shift would be a measure of the membrane potential, and the potential could facilitate delayed fluorescence in the manner that Crofts et al. [3] have proposed. Arguing against this interpretation, however, is the finding that the enhancement of delayed fluorescence is not markedly sensitive to gramicidin. One can develop the argument more quantitatively as follows. In the absence of gramicidin, the amplitude of the delayed fluorescence following the second of two flashes spaced 2 s apart is about 9-fold greater than that following the first flash (Fig. 1). Under similar conditions, the total amplitude of the carotenoid band shift is only about 30 % greater after the second flash than it is after the first (Fig. 6). The more dramatic change in the delayed fluorescence could be rationalized on the hypothesis [3, 5] that delayed fluorescence increases exponentially with the membrane potential, while the carotenoid band shift increases only linearly. But if this were the case, the collapse of the membrane potential to a value near zero would decrease delayed fluorescence to a level below that which one observes on individual, widely spaced flashes. This does not

happen. In the presence of enough gramicidin to make the carotenoid band shift undetectably small, the intensity of the delayed fluorescence with repetitive flashes spaced 2 s apart is still about nine times greater than that with widely spaced flashes (Fig. 2A).

It appears, therefore, that the enhancement of delayed fluorescence does not depend primarily on a membrane potential. In addition, because gramicidin has no detectable effect on the delayed fluorescence which follows widely separated flashes, it appears that any membrane potential that is generated immediately by the primary photochemical reaction itself does not influence delayed fluorescence significantly. On the other hand, the enhancement of delayed fluorescence is partially inhibited by gramicidin, and this would suggest that the state which gives rise to enhancement is somehow linked to transmembrane potential gradients, though perhaps indirectly. The interrelationships between delayed fluorescence, membrane potentials, the carotenoid band shift, and the "high energy" state which drives photophosphorylation thus continue to be far from clear, and the model proposed by Crofts et al. [3] would seem to be an oversimplification.

It is possible that the key step leading to the enhancement of delayed fluorescence is not the reduction of P^+X^- to PX^-, but another electron transfer reaction which rapidly follows this one. The electron donor which normally reduces P^+ in *Rps. sphaeroides* is cytochrome c_2 [8, 9], and measurements of absorbance changes at 420 and 550 nm indicated that the oxidation of a *c*-type cytochrome did occur after flash excitation at moderately low redox potentials (Fig. 8). However, little cytochrome accumulated in the oxidized form, presumably because re-reduction occurred at a rate comparable to the oxidation rate.

An indication that the re-reduction of the cytochrome might actually be the reaction of interest comes from the redox titrations of Fig. 5. The enhancement of delayed fluorescence appears to require the reduction of a component with an E_{m8} near +240 mV. This is lower than the E_m of cytochrome c_2. In its soluble form, the cytochrome has an E_m of +345 mV (independent of pH); the membrane-bound cytochrome has an E_m of +295 mV [9]. However, the significance of the discrepancy between the observed and expected E_m values is not clear. As we have emphasized above, the present experiments deal with chromatophores in which electron transfer is severely inhibited by the solubilization of cytochrome c_2 and the addition of *o*-phenanthroline. It is possible that *o*-phenanthroline causes shifts in the apparent E_m values of components other than X, and it is possible that components other than cytochrome c_2 and X^- transfer electrons to P^+. Dutton and Jackson [8] and Evans and Crofts [28] have suggested that a *b*-type cytochrome with an E_{m7} near +150 mV reduces P^+ in reaction centers which are depleted of cytochrome c_2. The redox buffers which are added for the titrations probably also can act as reductants for P^+, although the titrations probably do not simply measure the reduction of one of the buffers, because changing the buffers has little effect on the apparent E_m (Fig. 5). Finally, *Rps. sphaeroides* chromatophores contain a non-heme iron protein with an E_m of approx. +280 mV which could be involved in the reduction of P^+ or the re-reduction of cytochrome c_2 [29]. The iron undergoes oxidation when the chromatophores are illuminated [29].

Earlier workers who used phosphoroscopes equipped with continuous light sources appear to have observed the enhancement effect in *Rps. sphaeroides* chromato-

phores on several occasions, although the limitations of such instruments prevented them from distinguishing enhancement from changes in the basal level of delayed fluorescence. Thus, Fleischman and Clayton [11] noted the inhibitory effect of ionophores; Zankel [27] reported that reduced cytochrome *c* stimulated delayed fluorescence; and Sherman [10] found a stimulation by the redox dye DAD. The observations of Zankel [27] and Sherman [10] seem likely to reflect the dependence of the enhancement effect on proper poising of the redox potential.

Apart from a small inhibition by gramicidin, our earlier study of *Rps. viridis* did not give clear indications of the enhancement effect. Although a major reason for this probably was simply that all of the earlier measurements were made with widely spaced flashes, it would be difficult to measure delayed fluorescence from *Rps. viridis* chromatophores under conditions that would reveal enhancement. At low redox potentials, the membrane-bound cytochromes of *Rps. viridis* reduce P^+ so rapidly and efficiently that delayed fluorescence is not detectable with our present instrumentation [1].

Hopefully, improvements in the speed of the phosphoroscope will allow future work to explore the phenomenon of enhancement in a system which retains rapid cytochrome photooxidation. With faster instrumentation, it also should be possible to escape the need for *o*-phenanthroline to lengthen the lifetime of X^-. Refinements of this nature should help to clarify the interpretation of the present findings.

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