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DELAYED FLUORESCENCE FROM *RHODOPSEUDOMONAS VIRIDIS* FOLLOWING SINGLE FLASHES

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SUMMARY

Delayed fluorescence from *Rhodopseudomonas viridis* membrane fragments has been studied using a phosphoroscope employing single, short actinic flashes, under conditions of controlled redox potential and temperature. The emission spectrum shows that delayed fluorescence is emitted by the bulk, antenna bacteriochlorophyll. The energy for delayed fluorescence, however, must be stored in a reaction-center complex including the photooxidized form (P^+) of the primary electron-donor (P) and the photoreduced form (X^-) of the primary electron-acceptor. This is shown by the following observations: (1) Delayed luminescence is quenched (a) at low redox potentials which allow cytochromes to reduce P^+ rapidly after the flash, (b) at higher redox potentials which, by oxidizing P chemically, prevent the photochemical formation of P^+X^-, and (c) upon transfer of an electron from X^- to a secondary acceptor, Y. (2) Under conditions that prevent the reduction of P^+ by cytochromes and the oxidation of X^- by Y, the decay kinetics of delayed fluorescence are identical with those of P^+X^-, as measured from optical absorbance changes.

The main decay route for P^+X^- under these conditions has a rate-constant of approximately $10^3\ s^{-1}$. In contrast, a comparison of the intensities of delayed and prompt fluorescence indicates that the process in which P^+X^- returns energy to the bulk bacteriochlorophyll has a rate-constant of $3.7\ s^{-1}$, at 295 °K and pH 7.8. The decay kinetics of P^+X^- and delayed fluorescence change little with temperature, whereas the intensity of delayed fluorescence increases with increasing temperature, having an activation energy of 12.5 kcal · mol^{-1}. We conclude that the main decay route involves tunneling of an electron from X^- to P^+, without the promotion of P to an excited state. Delayed fluorescence requires such a promotion, followed by transfer of energy to the bulk bacteriochlorophyll, and this combination of events is rare. The activation energy, taken with potentiometric data, indicates that the photochemical conversion of PX to P^+X^- results in increases of both the energy and the

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Abbreviations: BChl and BChl*, light-harvesting (antenna) bacteriochlorophyll in its ground and lowest excited singlet states; E, redox potential; E_m, midpoint redox potential; P or P_{985}, P*, and P^+, primary electron donor in the photochemical reaction center in its ground state, in its lowest excited singlet state, and in its oxidized state; X and X^-, the primary electron acceptor in its oxidized and reduced states; Y, a secondary electron acceptor.

entropy of the system, by 16.6 kcal · mol^{-1} and 8.8 cal · mol^{-1} · deg^{-1}. The intensity of delayed fluorescence depends strongly on the pH; the origin of this effect remains unclear.

INTRODUCTION

Chloroplasts and photosynthetic bacteria can continue to re-emit light for milliseconds, and even seconds, following their exposure to a brief period of illumination. Among the theories which have been advanced to explain such "delayed" fluorescence are electron-hole recombination [1–3], triplet-triplet annihilation [4], and the reversal of electron-transfer at photochemical reaction centers [5, 6]. In the latter theory, the return of an electron from the reduced primary electron-acceptor to the oxidized electron-donor promotes the reaction center chlorophyll or bacteriochlorophyll to an excited singlet state. The migration of energy from the reaction center back to the bulk (antenna) chlorophyll or bacteriophyll then provides an opportunity for the bulk pigments to emit light by fluorescence. In support of this model, a mutant of *Rhodopseudomonas spheroides* that lacks photochemical reaction centers does not emit delayed fluorescence [7]. A reversal of the forward reactions of photosynthesis should consume whatever states or compounds the forward reactions generate. An interest in the high-energy states which ultimately drive photophosphorylation therefore has encouraged studies of the effects of uncouplers and inhibitors [8–10], salt gradients [11–13], pH gradients [14, 15], and electrical fields [3, 16] on delayed fluorescence yields.

Most previous studies of delayed fluorescence have employed phosphoroscopes which excited the sample repetitively with a chopped beam of light from a continuous source [17, 18]. Because the recovery time of the sample usually was long relative to the interval between the actinic exposures, the sample entered a quasi-equilibrium state which was difficult to define precisely. In addition, few researchers have attempted to control the redox poise of the sample, even though the components of the electron transport system are sensitive to chemical oxidation and reduction. As a result of these experimental limitations, there is little direct evidence that delayed fluorescence actually represents a reversal of the normal photochemical reaction. Similarly, there has been little evidence as to whether the effects of inhibitors, ionophores and uncouplers on delayed fluorescence occur at the level of the primary electron-transfer, or at secondary steps.

We have attempted to clarify some of these problems by exciting membrane fragments from *Rhodopseudomonas viridis* with single flashes, spaced far enough apart so that the sample could relax completely between exposures. We adjusted the redox potential appropriately during all experiments. In addition, we employed an inhibitor to block electron-transfer between the primary and secondary electron-acceptors, so that we could focus attention on the primary photochemical reaction and its reversal.

MATERIALS AND METHODS

Rhodopseudomonas viridis was grown in liquid medium (4 g Difco yeast extract+3 g Difco Bacto-peptone/l) under a light mineral oil film for three days.

Illumination was supplied by unfiltered 250 W heat lamps. The temperature was 31 °C. Membrane fragments normally were prepared by sonicating washed cells at 0 °C in 0.4 M sucrose/0.1 M Tris, pH 7.7 for four 1-min periods using a Branson Sonifier (Model LS-75) operating at 5 A. For proton uptake experiments, the cells were broken in 0.4 M sucrose/1 mM Tris/0.06 M KCl. For use in the gramicidin experiments only, one batch of membrane fragments was prepared by two passages of the bacteria through a French pressure cell operating at 10 tons/inch^2. In all cases, cell debris was removed by centrifugation at 17 000$\times g$ for 15 min and the membrane fragments collected by pelleting at 100 000$\times g$ for 90 min. The membranes were resuspended for storage in 40 % ethylene glycol/60 % (0.1 M) Tris at pH 7.2 or 0.1 M KCl for proton uptake experiments. They could be stored in the ethylene glycol mixture at −4 °C for at least a month without significant loss of photochemical activity. Dr. Breck Byers kindly examined thin sections of a sample of the membrane fragments for us by electron microscopy. The membranes appeared generally vesicular with diameters between 46 and 115 nm. Bacteriochlorophyll concentration was estimated from the absorbance at 1013 nm [19]. One batch of isolated membrane fragments was deionized with a Sephadex column, lyophilized, and extracted with petroleum ether for 2 h in a Soxhlet apparatus. The extraction appeared to be exhaustive.

Delayed fluorescence measurements were made in a phosphoroscope of our own construction. The excitation source was a Xe flash lamp (EG and G FX-76) operating at a repetition rate of approximately 1 min^{-1} and with a pulse width of about 10 μs. Light from the flash was projected through a Corning 2600 filter, 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, through a Corning 2550 filter and then to an RCA 7102 photomultiplier which was cooled with dry ice. An additional Corning 2600 filter was added to the photomultiplier for the measurements of Fig 9B and C. The photomultiplier anode current passed to an amplifier (Analog Devices 44J) providing a current-to-voltage transimpedance of 10^7 Ω, and the amplified signal was captured with a Biomation model 802 transient recorder. Information from the transient recorder was averaged in a homebuilt digital computer of average transients. A typical experiment involved averaging between 15 and 70 individual measurements. Several different shuttering arrangements were used in the phosphoroscope. The one used for the fastest measurements involved a single blade which was driven by a 1 hp motor operating at speeds up to about 20 000 rev./min.

For prompt fluorescent measurements, light from a 45 W tungsten lamp passed through a monochromator, a Schott KG-3 filter, a chopper, and then to the sample. Emission was detected at 90 °C through a combination of Corning 2550 and 2600 filters. A second monochromator was added on the detection side for the measurements of Fig. 1. The detection photomultiplier and its associated equipment were the same as those used for the delayed luminescence measurements. Comparisons with the emission of a reference lamp of known color temperature (Eppley Labs, Inc.) were used to correct the measured fluorescence emission spectra for the wavelength-dependence of the detector and monochromator. Absorbance changes following flash excitation were measured as described elsewhere [20].

Because membrane suspensions were somewhat turbid, critical absorbance

values were determined using an integrating sphere, with the measuring light duplicating the actinic light for the conditions under which the absorbance was desired.

All measurements of the sample (except in the integrating sphere and certain proton uptake experiments) were made using a cuvette that was equipped with Pt and calomel electrodes for monitoring the redox potential. The cuvette design and the potentiometric equipment were similar to that described elsewhere [21]. To minimize phosphorescence, the cuvette was fabricated of quartz. When filled with water, this cuvette gave no significant delayed fluorescence in the phosphoroscope. For all measurements except those of proton uptake, the membrane fragments were diluted in 0.1 M potassium phosphate buffer, with pH as indicated in the figure legends, PMS (100 μM, except when indicated otherwise) was added, and the sample was rendered anaerobic by bubbling with nitrogen. The Piericidin A, 5-n-pentadecyl-6-hydroxy-4,7-dioxobenzothiazole and 7-n-pentadecyl-6-hydroxy-5,8-quinolinequinone were gifts from Drs Karl Folkers and Thomas Porter of the University of Texas at Austin.

RESULTS AND DISCUSSION

Spectral analysis of delayed fluorescence

Because the delayed fluorescence from *R. viridis* is weak, a measurement of its emission spectrum requires some sacrifice of resolution. To compensate for the errors that this introduces, the spectrum can be compared to a spectrum of prompt fluorescence obtained under similar conditions. Fig. 1 shows that the emission spectrum of delayed fluorescence coincides within experimental error with that of prompt fluorescence. The emission maximum is near 1040 nm in both cases. It would appear that *R. viridis*, like other photosynthetic organisms [22, 23], emits delayed fluorescence from its bulk, light-harvesting BChl.

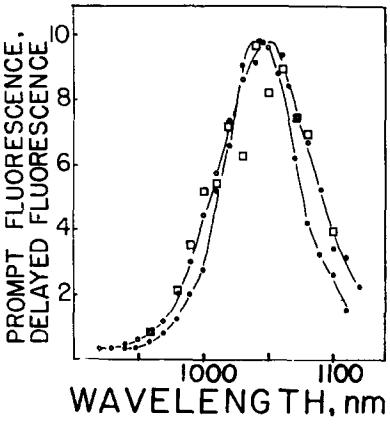

Fig. 1. Emission spectrum of *R. viridis* fluorescence. Delayed fluorescence (□) was measured with a monochromator bandpass of about 50 nm. Kinetic traces like that of Fig. 5 were replotted on a semilog grid and extrapolated to the time of the flash. Prompt fluorescence was measured with the same bandpass (○) and with a 10 nm bandpass. (●) Corrections were made for photomultiplier and monochromator sensitivity using a black-body radiation source of 2700 °K. BChl, 3.2 μM; *o*-phenanthroline, 1 mM; pH 7.8; *E*, 410 mV; temperature, 20 °C.

Dependence of delayed fluorescence on the redox potential

The circles in Fig. 2 show the intensity of delayed fluorescence as a function of the redox potential. The squares represent the amount of reaction center bacteriochlorophyll (P_{985}, or P) that remains photooxidized shortly after the actinic flash. The triangles in the same figure represent the amount of photooxidation of the high potential cytochrome C-558 in a parallel experiment. Open symbols describe measurements at 20 °C, filled symbols, measurements at 4 °C. At redox potentials well below the E_m of cytochrome C-558 (+ 0.33 V, [24]) both the cytochrome and P are in their reduced states prior to the flash. Following the photooxidation of P (to P^+), electron transfer from the cytochrome to P^+ occurs with a half-time of approximately 1 μs [25]. The comparatively slow measurements in Fig. 2 record the cytochrome photooxidation, but show little P^+ remaining after the flash. Cytochrome photooxidation decreases as the potential is raised above the E_m of the cytochrome, resulting in increased net yields of P^+ between +280 mV and +420 mV. As the potential is increased still higher, chemical oxidation of P depletes the amount of the reaction center bacteriochlorophyll that is available for photooxidation. The curve showing P photooxidation thus decreases above +420 mV [the E_m of P_{985} is +0.39 to +0.45 V [26]). The intensity of delayed fluorescence (measured at 1 ms after the flash) increases between +280 mV and +420 mV and decreases again between +420 mV and +520 mV (Fig. 2). Clearly, delayed fluorescence is related to the amount of photooxidized P that remains after the flash. The exact relation between the two is discussed more quantitatively below.

A second point shown by the curves in Fig. 2 is the apparent insensitivity of the E_m values of C-558 and P to temperature. Potentiometric titrations with *Chromatium vinosum*, in contrast, have shown that the E_m values of cytochrome C-555, P_{870}, and the primary electron-acceptor all depend strongly on the temperature [27].

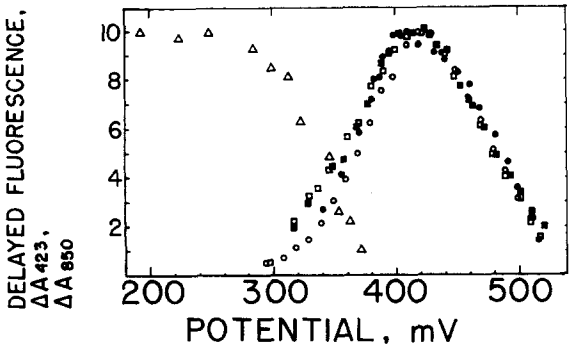

Fig. 2. Potentiometric titrations of cytochrome C-558, P, and delayed fluorescence. Cytochrome oxidation (△) measured at 432 nm following an Xe flash in the near IR (Corning 2600 filter). P photooxidation (□, ■) measured at 850 nm following a blue-green Xe flash (Schott BG-18 filter). Delayed fluorescence (○, ●) measured at 1 ms following an Xe flash in the near IR. Open symbols refer to titrations at 20 °C; filled symbols, 4 °C. Titrations were performed using small additions of potassium ferricyanide (6 mg/ml) and a saturated calomel-Pt electrode pair continuously monitored the solution potential. The full scale of 10 on the graph represents $2.1 \cdot 10^{-3}$ ΔA (□, ■); $5.3 \cdot 10^{-3}$ ΔA (△); 4.1 V (○); and 2.8 V (●). BChl, 4 μM (△), 3.2 μM (□, ■, ○, ●); pH 7.8; *o*-phenanthroline, 0.4 mM (△), 1.0 mM (□, ■, ○, ●).

Dependence of delayed fluorescence on electron-transport inhibitors

The requirement for P photooxidation (in contrast to chemical oxidation) presumably reflects a parallel requirement for an electron on the primary acceptor, X. Electron-transport inhibitors which prevent escape of electrons from X^- to secondary acceptors should increase the yield of delayed fluorescence. *O*-phenanthroline is such an inhibitor [28], and Fleischman and Clayton [8] have shown that *o*-phenanthroline does increase delayed fluorescence in *R. spheroides* and *R. viridis*. Fig. 3 shows the effect of *o*-phenanthroline on the intensity of delayed fluorescence, as measured at 1 ms under our experimental conditions (open squares). The delayed fluorescence that is plotted is only the amount increased by *o*-phenanthroline; the control yield (without inhibitor) was subtracted from each data point. In this experiment, the amount of delayed fluorescence without the inhibitor was about 8 % of the amount in samples with saturating concentrations of *o*-phenanthroline. At times closer to the flash, the effect of *o*-phenanthroline on delayed luminescence was less pronounced (see below).

The closed circles in Fig. 3 show parallel measurements (at lower redox potential) of the inhibition of cytochrome photooxidation on the second of two, closely-spaced, saturating laser flashes. The amount of cytochrome oxidation on the second flash is a measure of the ability of X^- to transfer an electron to a secondary acceptor, Y, in the interval between the two flashes (see below and ref. 20). Inhibition of the electron-transfer reaction by *o*-phenanthroline is not complete, but rather saturates at about 88 % blockage.

Fig. 3 shows that the inhibition of the secondary electron-transfer reaction and the enhancement of delayed fluorescence parallel one another. Other inhibitors of the secondary electron-transfer, including Piericidin A, 5-n-pentadecyl-6-hydroxy-4,7-dioxobenzothiazole and 7-n-pentadecyl-6-hydroxy-5,8-quinolinequinone [29] also

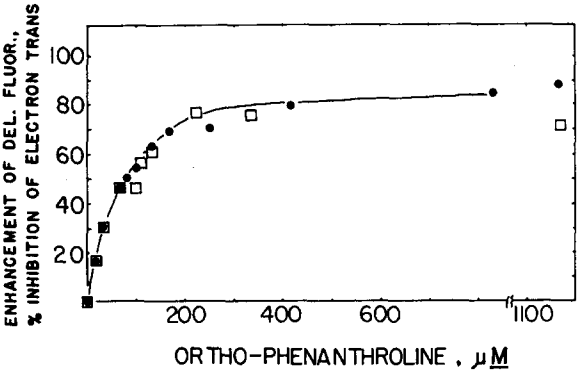

Fig. 3. Inhibition of secondary electron-transfer and enhancement of delayed fluorescence by *o*-phenanthroline. The secondary electron-transfer (X^--to-Y) reaction was measured by two saturating Q-switched ruby laser flashes spaced 8 ms apart. The calculated inhibition (●) represents:

$$100\,\% - \left(\frac{\text{second flash cytochrome oxidation with inhibitor}}{\text{second flash cytochrome oxidation without inhibitor}}\right) \times 100.$$

The open squares (□) represent normalized enhanced luminescence at 1 ms calculated as: luminescence with inhibitor −luminescence without inhibitor. BChl, 1.8 μM; PMS,75 μM; pH 7.8; *E*, 240 mV (●); 415 mV (□).

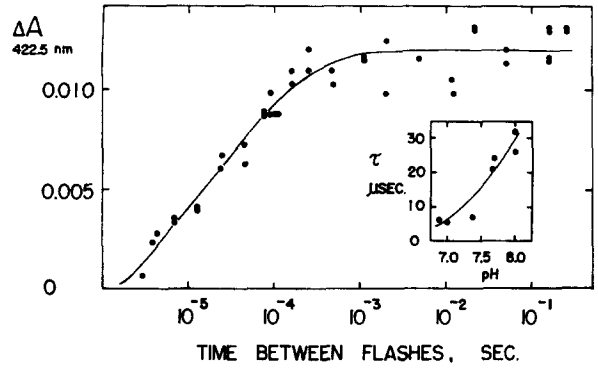

Fig. 4. Kinetics of the secondary electron-transfer from X^- to Y. The main part of the figure shows the amount of cytochrome photooxidation (ΔA 422.5 nm) on the second of two saturating laser flashes, as a function of the length of the delay between the flashes. Solution pH 7.65; PMS. 7 μM; *E*, approx. 250 mV. The insert shows the delay that was required for the second flash to have half its maximal effect, as judged from plots like those of the main part of the figure. The half-time does not characterize the kinetics fully, because they are not first-order.

were effective at increasing delayed fluorescence. Extraction of lyophilized membrane fragments with petroleum ether had an effect similar to that of the electron-transfer inhibitors and *o*-phenanthroline had no additional effects on these membranes. Such extraction has been shown to prevent the X^--to-Y reaction in *C. vinosum* chromatophores [29]; the extraction evidently removes Y, which appears to be ubiquinone [29].

The stimulation of delayed fluorescence by treatments which inhibit electron-transfer between X^- and Y implies that the electron-transfer reaction normally occurs very rapidly after the flash. Fig. 4 shows more direct evidence on this point. The main part of the figure shows the amount of cytochrome C-558 photooxidation on the second of two flashes, as a function of the length of time separating the two flashes. Because each photosynthetic unit contains two cytochrome C-558 hemes [26], and because electron-transport from one of the hemes to P^+ is essentially complete within 3 μs after the first flash, the rate at which the system develops the capacity to perform cytochrome oxidation on the second flash probably depends primarily on the rate at which X^- returns to the oxidized state after the flash. Fig. 4 gives a half-time of about 24 μs for the reaction at pH 7.65; the insert shows that the reaction rate increases with decreasing pH. The rates are about five to ten times greater than those which occur in *C. vinosum* chromatophores [20].

Decay kinetics of P^+ and delayed fluorescence

Fig. 5 shows the kinetics of delayed fluorescence decay as a solid line and the rapid phase of P^+ decay superimposed as open circles. For these measurements, the redox potential was high enough to oxidize cytochrome C-558 almost completely, preventing the cytochrome from reducing P^+ after the flash. *O*-phenanthroline was present to block electron-transport between X^- and Y. Conditions therefore were optimal for a back-reaction between X^- and P^+. Because the *o*-phenanthroline block is incomplete (Fig. 3), electrons escape from X^- in some photosynthetic units. In these, the return of the electron to P^+ must follow a slower and less direct route. The faster decay of P^+ by the direct back-reaction thus accounts for only about 88 % of

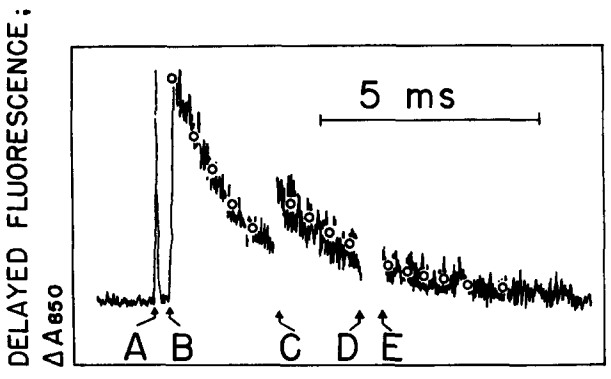

Fig. 5. Decay kinetics of P^+ and delayed fluorescence. The noisy solid line shows the decay of delayed luminescence. The actinic flash occurs at A. The phosphoroscope window opens at B. At C the amplification is increased 2-fold. (Separate measurements with different amplifications have been replotted together for the figure). The phosphoroscope measuring window briefly closes at D and reopens at E. The open circles represent the amount of P^+ at various times after the flash, after subtraction of the 12 % of the initial P^+ which decays by a route that is insensitive to *o*-phenanthroline (see text and Fig. 3). P^+ was measured from the blue shift of the 830 nm absorption band. Letters A and C also apply to P^+. The delayed luminescence peak B at is about 0.7 V and $\Delta A\ P^+$ is about $4.9 \cdot 10^{-3}$ at B. BChl, 3.4 μM; *o*-phenanthroline, 1 mM; room temperature; *E*, 420 mV.

the recovery and this is the quantity plotted in Fig. 5 as open circles. Whereas the redox titrations in Fig. 2 indicate a general dependence of delayed fluorescence on the amount of photo-oxidized $P_$ the more discriminating kinetic comparison of the two in Fig. 5 shows that they are directly proportional to one another within experimental error. Fleischman [30] has reached the same conclusion using quite different experimental techniques.

The half-time of decay of delayed fluorescence and P^+ is approximately 0.6 ms, but the kinetics do not follow a single exponential expression. The sum of at

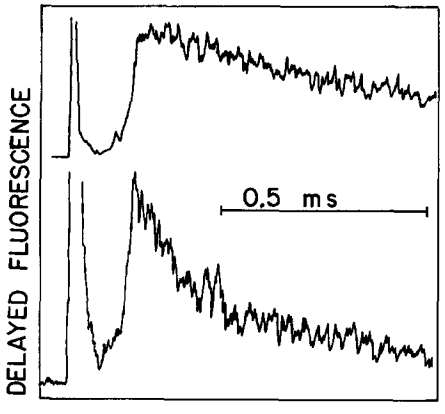

Fig. 6. Comparison of delayed luminescence in the presence and absence of *o*-phenanthroline. The upper trace shows decay of luminescence in a sample treated with 1 mM *o*-phenanthroline. The lower trace is amplified 12.5 times with respect to the upper and shows decay of the luminescence in the absence of inhibitor. pH 8.0; BChl, 2.6 μM; *E*, 418 mV; temperature, 23 °C.

least three different exponentials is required to describe the curves over about three decades of decay. Because the decay kinetics are sensitive to the pH (see below), the complexity of the decay could arise from a large number of microscopic states which differ in the degree of protonation.

Although the decay kinetics of delayed fluorescence match those of P^+ under the conditions of Fig. 5, their proportionality breaks down under other circumstances. Such circumstances include situations which allow proton uptake or the maintenance of transmembrane electrical fields (see below), or conditions that permit secondary electron transfer from X^- to Y. Fig. 6 shows the decay kinetics of delayed fluorescence under the last of these conditions. In the first 0.2 ms after the flash, the delayed fluorescence decays much more rapidly in the absence of *o*-phenanthroline than it does in the presence of the inhibitor. The kinetics of the initial decay are comparable to those of the secondary electron-transfer reaction (Fig. 4). Presumably, the intensity of the delayed luminescence at even earlier times would be the same in the presence or absence of *o*-phenanthroline, but our phosphoroscope was not fast enough to allow a test of this point.

The effect of pH on delayed fluorescence

The closed squares in Fig. 7, show the pH-dependence of the delayed luminescence, as measured by extrapolation to the time of the actinic flash. Open squares represent prompt fluorescence in a parallel experiment. Open circles in the same figure show the amount of P^+ generated at the time of the flash, and the amount of P^+ remaining at 7 ms is shown by closed circles. The intensity of delayed fluorescence depends quite strongly on the pH with the maximum occurring near pH 8. The variation of delayed luminescence with pH is not due solely to changes in the yield of prompt fluorescence, because this yield changes in the opposite direction between pH 5 and 8; it is not due to changes in the inhibitor activity because P^+ recovery is not affected. The Arrhenius activation energy for luminescence does not vary greatly with

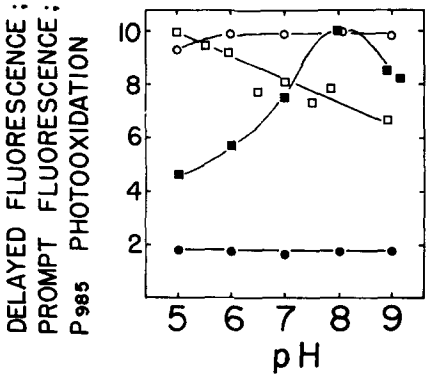

Fig. 7. Effect of pH on delayed light emission, prompt fluorescence emission and P photooxidation. Closed squares (■) show a three experiment average of delayed luminescence (extrapolated to the time of the flash) as a function of pH. Open squares (□) show prompt fluorescence excited by continuous 606 nm illumination. Total P^+ measured from ΔA at 444 nm at the time of the flash is shown by open circles (○) and at 7 ms after the flash by closed circles (●). Full scale of the figure represents about 0.0027 ΔA, 95 mV prompt fluorescence, and 2.6 V delayed luminescence. BChl, 3.1 μM (■), 5.1 μM (□), 4.1 μM (○, ●); *o*-phenanthroline, 1 mM; *E*, 420 mV.

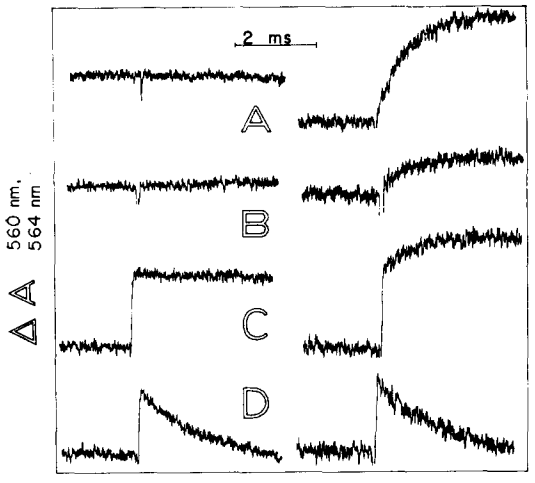

Fig. 8. Proton uptake by unbuffered membrane fragments. Experiments on the left show flash-induced absorbance changes of the membrane fragments before, and on the right, after the addition of the pH indicator, phenol red. All solutions were 6.3 μM BChl; 0.1 M KCl at pH 7.0; phenol red (right side of figure), 0.02%. A E, 240 mV; no o-phenanthroline. B E, 240 mV; 1 mM o-phenanthroline. C E, 420 mV, no o-phenanthroline. D E, 420 mV; 1 mM o-phenanthroline. A and B measured at 564 nm; C and D measured at 560 nm. An upward deflection is an absorbance increase.

pH (see below), so the effect of pH on delayed luminescence must be due mainly to entropic factors.

The pH-dependence of the delayed fluorescence could reflect the rapid uptake or release of protons following the flash. In order to evaluate this possibility, we measured pH changes in the solution following flash activation of unbuffered membrane fragments. To relate the amount of proton uptake to the amount of P^+ produced by the flash, cytochrome C-558 photo-oxidation was measured in parallel experiments. Additions of standardized NaOH were used for calibration. Fig. 8 shows the results of proton uptake measurements under various conditions. The traces on the right hand side of the figure represent absorbance changes due to the pH indicator, superimposed on absorbance changes due to components of the membrane fragments themselves; the traces on the left are control measurements of the absorbance changes due to the membrane components alone. Expt A represents samples poised at a redox potential of +240 mV. It exhibits an uptake of about 0.68 protons per photooxidized reaction center with a half-time of about 510 μs. Experiment B featured samples at +240 mV and treated with o-phenanthroline. Under these conditions, the proton uptake decreased to about 0.23 protons per P^+; the half-time remained essentially unchanged. Expt C represents samples at +420 mV; the proton uptake was about 0.25 protons per P^+, with the half-time remaining at about 500 μs. Treatment of the membrane fragments at +420 mV with o-phenanthroline (Expt D) abolishes all proton uptake. Because most measurements of delayed luminescence were made at high redox potential in the presence of o-phenanthroline, proton uptake during the luminescence observations is unimportant. We conclude that the variation of the delayed fluorescence with pH reflects protonic equilibria that are established prior to the flash, rather than the uptake of protons following the flash.

The decay kinetics of delayed luminescence also change with pH. At pH 6.0, 7.0, 8.0 and 9.0, the initial decay of the luminescence has half-times of about 0.79, 0.64, 0.54, and 0.52 ms, respectively.

Effects of ionophores on delayed luminescence

Crofts, et al. [31] have suggested that P^+ and X^- form an electric dipole that is aligned perpendicular to the membrane surface. If this is correct, a transmembrane electrical field would either favor or hinder the reversal of the photochemical separation of charge across the membrane, depending on the direction of the field. In order to test this theory, we examined the effect of gramicidin D on delayed fluorescence. By providing a highly conductive transmembrane pore [32], gramicidin should dissipate energy that might be stored in transmembrane electric fields after the flash.

Gramicidin had no measureable effect on delayed luminescence with membrane fragments which were produced by sonication. Because Jones et al. [33] have reported that preparations obtained from *R. viridis* by the use of a French press perform photophosphorylation, we also prepared a sample of membrane fragments using the French press. This preparation exhibited a small inhibition of delayed fluorescence by 5 μM gramicidin. Extrapolating to the time of the flash, the extent of the inhibition was about 20 %. The difference between the delayed fluorescence of the control sample and that of the sample treated with gramicidin decayed following an exponential curve with a half-time of about 0.9 ms. All of the experiments that are described elsewhere in this report involved preparations that were made by sonication.

The rate of the luminescent back-reaction

The preceding sections show that the process which leads to delayed fluorescence is kinetically first-order in the concentration of P^+X^-, although the apparent rate constant depends on several variables, including the pH. These results support the conclusion that delayed fluorescence represents a reversal of the photochemical transfer of electrons from P to X. Following its formation, P^+X^- decays with a half-time of 0.6 ms. The question therefore arises whether the process that results in delayed fluorescence makes a major contribution to this decay, or whether it is only a minor competitor with another, non-radiative decay path. To obtain a quantitative measure of the rate of the luminescent path, we compared the intensity of the delayed fluorescence with the intensity of prompt fluorescence. Fleischman [30] has pointed out that such a comparison provides an indication of the rate at which singlet excitons are injected from the reaction center back into the pool of bulk bacteriochlorophyll, relative to the rate at which excitons are introduced to the bacteriochlorophyll by the direct absorption of light.

We first estimated the concentration of P^+ produced by the phosphoroscope flash by measuring the amount of cytochrome photooxidation that followed the flash (at a redox potential of +240 mV). This seemed preferable to an attempt to measure P^+ directly because the signal-to-noise ratio is higher for cytochrome measurements and the $\Delta\varepsilon$ is better characterized. Fig. 9A shows the result as a function of the flash strength. Assuming a differential extinction coefficient of $\Delta\varepsilon_{424} \approx 10^5 \cdot M^{-1} \cdot cm^{-1}$ for cytochrome oxidation [34], the quantum yield is 0.48. We take 0.48 also to be the quantum efficiency for the initial photooxidation of P, because the cytochrome oxidation is linked to essentially complete reduction of the P^+ (Fig. 2).

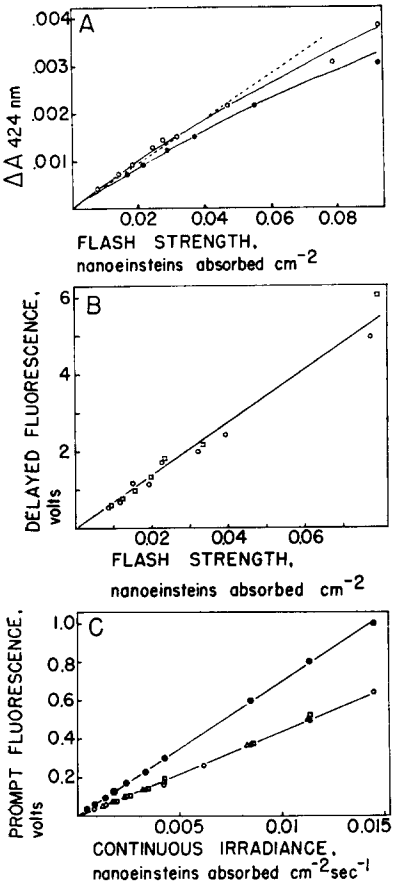

Fig. 9.A Quantum requirement for cytochrome oxidation. The absorbance change measured at 424 nm followed a Xe flash (Corning 2600 filter) is plotted for two different samples (○, ●) as a function of the amount of light absorbed from the Xe flash. The incident flash was measured with a calibrated SGD 100 (EG and G) photodiode and calibrated neutral density filters were employed to obtain the data points. Absolute absorbance of the sample was measured in an integrating sphere using a similar Xe flash. B Delayed luminescence for two different experiments (○, □) is plotted as in A. C Prompt fluorescence, plotted as a function of the rate of absorption of 606 nm continuous illumination. The detection optics and the photomultiplier voltage and filters (Corning 2600 and 2550) were identical in B and C. BChl, 2.7 μM; *o*-phenanthroline, 1 mM (○, A; ●, A; ○, B; □, B; ○, C); *E*, −400 mV (●, C); +240 mV (○, A; ●, A; △, C); +400 mV (○, C; □, C); and +420 mV (○, B; □, B).

This agrees with Thornber et al. [26], who reported a quantum efficiency of 40–50 % for P photooxidation in a reaction center preparation from *R. viridis*. The concentration of P^+X^- remaining after the flash (in nmol · cm^{-2}) is calculated by multiplying the incident irradiance by 0.48, 1-T (the absolute absorbance) and 0.88 (the extent of *o*-phenanthroline inhibition of the X^--to-Y reaction).

Fig. 9B shows the delayed fluorescence as a function of flash intensity. For this figure, the delayed fluorescence was extrapolated to the time of the flash. A comparison of Figs 9A and 9B confirms that the initial intensity of the delayed fluorescence is directly proportional to the concentration of P^+X^-. The instrumental proportionality

constant, which we require below, is $1.61 \cdot 10^2$ V · (nmol $P^+X^-/cm^2)^{-1}$.

The relationship between prompt fluorescence and the photon absorption rate was determined in several samples of membrane fragments under various conditions. Fig. 9C shows the results. The excitation source was a chopped continuous beam of monochromatic (606 nm) light in all cases and the strength of the prompt fluorescence signal was comparable to the strength of the delayed fluorescence signals with which we wished to compare it. The rate of production of excitons in the bulk-bacteriochlorophyll was calculated by multiplying the incident irradiance by the absolute absorbance (1-T), since the 606 nm light was absorbed directly by the bulk bacteriochlorophyll. Closed circles in Fig. 9C represent a sample reduced to −400 mV with dithionite; open circles, a sample at +400 mV treated with *o*-phenanthroline; open squares, a sample at +400 mV without inhibition; and open triangles, a sample at +240 mV without inhibitor. All of these measurements employed comparatively weak excitation beams and the fluorescence intensity was a linear function of the excitation intensity in all cases. The quantum yield for fluorescence, Φ_F, did not change upon oxidation of the cytochromes or inhibition of the secondary electron-transport reaction, presumably because the initial photooxidation of P still occurred with a high quantum yield under these conditions. Strong reduction of the sample did increase Φ_F, presumably because the reduction of X then prevented the photochemical electron transfer. The observed 1.6-fold increase in Φ_F when photochemistry is prevented is slightly smaller than the 1.9-fold change expected from the expression $\Phi_C = 1 - \Phi_{F1}/\Phi_{F2}$, where Φ_C is the measured quantum yield for photochemistry (0.48), Φ_{F1} the fluorescence quantum yield with photochemistry occurring and Φ_{F2} the fluorescence yield with photochemistry blocked.

The instrumental proportionality constant relating prompt fluorescence to the rate of generation of excitons in the bulk bacteriochlorophyll (Fig. 9C, open circles) is 43.6 V · (nmol BChl*/cm^2/s)$^{-1}$. This number is based on the lower fluorescence yield that we measured in the samples at +240 and +400 mV. The prompt fluorescence yield in the delayed luminescence experiments of Fig. 9B probably was comparable to the lower of the two yields shown in Fig. 9C, because the delayed fluorescence measurements involved flashes that were too weak to approach saturation of the photochemical apparatus.

Because the detector cannot distinguish between prompt and delayed fluorescence (Fig. 1), the apparent first-order rate constant, k_e, for the production of bacteriochlorophyll excitons from P^+X^- is $161/43.6 = 3.7\ s^{-1}$. The calculated rate for the back-reaction that leads to luminescence is thus slower than the observed decay rate of P^+ (Fig. 5) by more than two orders of magnitude. This means that delayed fluorescence is not the major relaxation pathway for P^+X^-. This conclusion is supported by studies of the temperature dependence of delayed fluorescence and P oxidation (see next section).

Our value for the rate constant of the luminescent back-reaction is about 10^3 times greater than the value which Fleischman [30] has calculated using a similar analysis. Fleischman's value is $1 \cdot 10^{-3}$ for −23 °C; using his activation energy of 4.15 kcal/mol allows an extrapolation to $3.6 \cdot 10^{-3} \cdot sec^{-1}$ at 23 °C. His calculations rest on measurements that were made in the absence of *o*-phenanthroline, at uncontrolled (presumably lower) redox potentials, at longer times after illumination, and at temperatures below 0 °C.

The rate constant k_e that we have calculated here describes the rate at which the reversal of the photochemical electron-transfer reaction returns excitons to the bulk bacteriochlorophyll. This is not the same as the actual rate of delayed fluorescence, nor is it identical with the rate of the reversal of the electron-transfer reaction. Reversing the electron-transfer reaction promotes the reaction-center complex itself to an excited singlet state, P*X, but we do not know what fraction of the complexes that reach this state transfers energy to the bulk bacteriochlorophyll, and what fraction reenters the forward electron-transfer reaction, regenerating P^+X^-. To make the discussion more concrete, we can symbolize the reactions that are involved as follows:

(1) $h\nu + \mathrm{BChl} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{BChl}^*$

(2) $\mathrm{BChl}^* + \mathrm{PX} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{BChl} + \mathrm{P}^*\mathrm{X}$

(3) $\mathrm{P}^*\mathrm{X} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{P}^+\mathrm{X}^-$

The rate constant that we have measured is

$$k_e = k_{-3}k_{-2}(k_{-2}+k_3)^{-1}.$$

Unfortunately, we do not know enough about the relative magnitudes of k_{-2} and k_3 to allow a calculation of k_{-3} from k_e.

Whereas k_e must be smaller than k_{-3}, the rate-constant for the actual emission of delayed fluorescence is in turn smaller than k_e, by a factor of the fluorescence yield. In other words, the reversal of the electron transfer reaction may occur many times, before an exciton is returned from P* to the bulk bacteriochlorophyll, and the return of energy to the bulk bacteriochlorophyll may occur many times before the bacteriochlorophyll fluoresces.

Dependence of delayed fluorescence on temperature

The temperature-dependence of delayed fluorescence is shown in Fig. 10, with closed circles representing membrane preparations treated with *o*-phenanthroline and open triangles representing preparations extracted with petroleum ether. Delayed fluorescence increases with increasing temperature and the slope is the same for both preparations. Fig. 11 shows that under the conditions similar to those used in Fig. 10 for the unextracted membrane fragments, neither the amount of P photooxidized by the flash nor the amount that recovers by the fast back-reaction is a function of temperature. The temperature-dependence of delayed fluorescence in Fig. 10 therefore can only be explained by an activation energy. The Arrhenius activation energy is 12.5 kcal · mol^{-1} at pH 8. The activation energy is not strongly dependent on the pH; similar measurements at pH 5, 6, 7, and 9 gave values of 13.9, 14.6, 12.8, and 12.2 kcal · mol^{-1}, respectively.

Although the intensity of the delayed fluorescence depends on the temperature and is proportional to the amount of photooxidized P, the decay kinetics of P^+ and delayed luminescence change only slightly with the temperature (Fig. 11). This agrees with the conclusion drawn from the rate constants that delayed fluorescence cannot

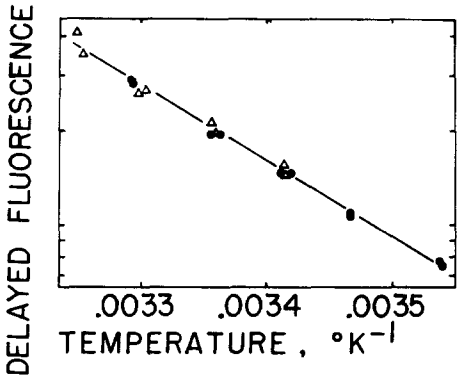

Fig. 10. Arrhenius plot for delayed luminescence extrapolated to the time of the flash. Open triangles (△) indicate luminescence from membrane fragments extracted with petroleum ether; closed circles (●), luminescence from unextracted membrane fragments inhibited with 1 mM *o*-phenanthroline. The delayed fluorescence is plotted in logarithmic form and the temperature as a reciprocal. BChl, 5.3 μM; pH 8.0; *E*, 420 mV. Each data point represents a fresh sample.

be a quantitatively important pathway in P^+ relaxation. If it were important, the P^+ decay kinetics would also depend strongly on the temperature. Presumably, temperature-independent electron tunneling is the major decay path for P^+X^-. This route must afford PX directly, without the promotion of the complex to P*X. Fleischman [30] recently has described experiments that point to the same conclusion from a different experimental approach. The activation energy of 4.15 kcal · mol^{-1} that he reported for delayed fluorescence is substantially lower than our value.

The activation energies given above are not influenced by a small increase in the decay rates that does occur with increasing temperature, because the mea-

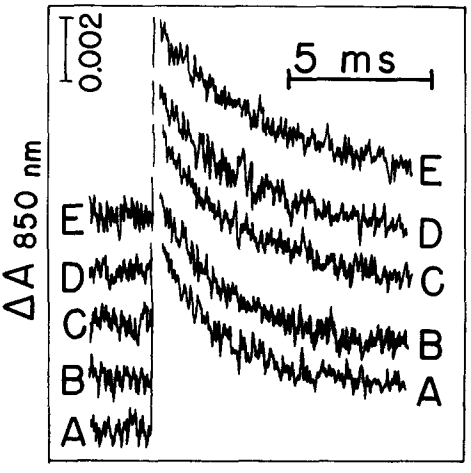

Fig. 11. P photooxidation as a function of temperature. Five different experiments are shown displaced above one another so that the times of the flash coincide. The absorbance change was measured at 850 nm. BChl, 5.8 μM; *o*-phenanthroline, 1 mM; pH 7.8, *E*, 410 mV; temperature: A, 19.30 °; B, 13.8°; C, 9.5°; D, 5.5°; and *E*, 0.0° C.

surements all include an extrapolation to the time of the flash. At later times, the apparent activation energy is somewhat smaller (about 2 kcal · mole^{-1} lower for 1 msec delayed light). The small change in the decay kinetics with temperature could be due to a variation of the pK values of components of the membrane.

The activation energy for delayed fluorescence will be equivalent to that for the reversal of photochemistry if $k_{-2}(k_{-2}+k_3)^{-1}$ does not vary significantly with temperature. This assumption probably is valid, because P photooxidation occurs at temperatures as low as 77 °K [35].

In order to measure the activation energy of the back-reaction that leads to luminescence, it was necessary to inhibit forward electron-transfer between X^- and Y. If the secondary forward reaction had been allowed to proceed, its temperature-dependence [20] would have obscured that of the back-reaction. In addition, a temperature-dependent proton uptake might alter the probability of the luminescent back-reaction [31, 36, 37]. Unfortunately, inhibitors such as *o*-phenanthroline could alter the properties of X, perturbing both the kinetics and the thermodynamics of the back-reaction. There have, in fact, been reports that *o*-phenanthroline shifts the apparent E_m of X by +130 mV in chromatophores of *R. viridis* [24] and *C. vinosum* [38, 39]. However, we feel that the interpretation of these reports requires caution. The photosynthetic apparatus returns to equilibrium extremely slowly after illumination at low redox potentials in the presence of *o*-phenanthroline. In *C. vinosum* chromatophores, *o*-phenanthroline does not interfere with the photooxidation of cytochrome C-552 by single flashes at low redox potentials (−25 mV) [21], but we have found that one must allow many minutes for recovery in order to repeat the measurement (unpublished observations). Incomplete relaxation after illumination could lead to an erroneous impression that a decrease in photochemical activity had resulted from a shift of the E_m of X. The following observations suggest that inhibition of the X^--to-Y reaction with *o*-phenanthroline actually does not cause a major change in the properties of X. (1) The activation energy for the production of delayed fluorescence is the same for membrane fragments that are treated with *o*-phenanthroline as it is for preparations in which the secondary electron-transfer reaction has been blocked by lipid extraction (Fig. 10). (2) *O*-phenanthroline does not alter the delayed luminescence in the extracted membrane fragments. (3) The kinetics of the back-reaction between P^+ and X^- are essentially the same in the presence of *o*-phenanthroline or other inhibitors of the X^--to-Y reaction as they are if one prevents the reaction by extracting the ubiquinone (see also ref. 29). However, we do not view these arguments as being entirely conclusive. It is possible that the effect of *o*-phenanthroline on X is simply below the limits of resolution of the present experiments.

The experimental data now available allow the following thermodynamic discussion of the photochemical electron-transfer reaction in *R. viridis*. We shall consider only data from vesicles in which transmembrane electrical potentials are insignificant (as judged from the insensitivity of the delayed luminescence to gramicidin), because quantitative analysis of electrical field effects would depend upon knowing what portion of the vesicle population is sealed. The energy of the lowest-lying excited singlet state of P_{985} (P*X) is about 28.6 kcal · mol^{-1} above that of the ground state, assuming that the reaction center complex would fluoresce at approximately 1000 nm if photochemistry did not intervene. The activation enthalpy for delayed fluorescence ($\Delta H^\dagger$ = Ea-RT) is about 12 kcal · mol^{-1} at pH 7.8. This implies

that the product of the primary electron-transfer reaction (P^+X^-) stores enthalpy in the amount of approximately 16.6 kcal · mol^{-1} relative to the ground state (PX), if one makes two assumptions. First, one must assume that there is no activation energy for the forward reaction ($P^*X \rightarrow P^+X^-$). This assumption seems safe from the observation [35] that P photooxidation occurs at very low temperatures. The second assumption is that the conversion of P^+X^- to P*X in the reversal of the photochemical reaction occurs in a single step (Eqn 3). If more than one step is required, the activation energy will be that of the rate-limiting step, rather than the total energy change in the complete process. This assumption does not rest on firm ground.

The free energy of P^+X^- relative to that of PX can be calculated from potentiometric titrations of P and X. Taking $E_{m\,7.8}$ of P as +0.480 V (Fig. 2) and $E_{m\,7.8}$ of X as −0.125 V [24] and using $\Delta G^0 = -nF\Delta E_m$ gives 14.0 kcal · mol^{-1}. The fact that the free energy increase upon conversion of PX to P^+X^- is less than the enthalpy increase calculated above would imply that the entropy of P^+X^- exceeds that of PX by about 8.8 cal · mol^{-1} · deg^{-1}. This conclusion conflicts with the results of potentiometric and calorimetric measurements in *C. vinosum* chromatophores, which have suggested that the product of the photochemical reaction stores little or no energy. Instead, the free energy that is captured in the electron-transfer reaction in *C. vinosum* has appeared to take the form of a decrease in entropy [40]. The reason for this disagreement is unlikely to lie simply in differences between the two species, because preliminary measurements have indicated that the activation energy for delayed luminescence in *C. vinosum* is similar to that in *R. viridis*. The origin of the discrepancy could lie in the proton uptake which follows the flash under the conditions of the potentiometric and calorimetric measurements. Another possibility is that the second assumption stated above is incorrect. Reasons for suspecting that this may be the case have come from recent studies of the mechanism of the primary electron transfer reaction. In reaction center preparations from *R. spheroides*, the conversion of P*X to P^+X^- appears to involve at least two steps (W. W. Parson, R. K. Clayton and R. J. Cogdell, submitted for publication).

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