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THE REACTION BETWEEN PRIMARY AND SECONDARY ELECTRON ACCEPTORS IN BACTERIAL PHOTOSYNTHESIS

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SUMMARY

Following a 20-nsec actinic flash, which causes oxidation of P870 and cytochrome C422, *Chromatium* chromatophores enter a refractory state. While the chromatophores are in this state, a second flash does not cause further oxidation of P870 or cytochrome C422. The quanta of the second flash are wasted as fluorescence (and heat); apparently they do not energize an alternative photochemical reaction. The refractory state probably reflects the accumulation of the primary electron acceptor in a reduced form. By following the reappearance of the capacity for photochemistry, one can measure the kinetics of electron transfer between the primary electron acceptor and the secondary agent which reoxidizes it. In *Chromatium* chromatophores, this process requires about 60 μsec to proceed half-way to completion at pH 7, and 80 μsec at pH 8. The rate of the reaction increases with decreasing pH, but not in direct proportion to the proton concentration. It increases with temperature, with an E_a of about 8.3 kcal/mole. The kinetics are approximately second order in the concentration of the reduced acceptor.

INTRODUCTION

The primary photochemical reaction of bacterial photosynthesis is the oxidation of a special bacteriochlorophyll trimer[1] called P870, which REED AND CLAYTON[2] recently have isolated from the bulk bacteriochlorophyll. P870 oxidation occurs with a quantum yield near 1.0, in less than 0.5 μsec after a short actinic flash[3]. It probably occurs as rapidly as an exciton reaches P870, after the bulk bacteriochlorophyll absorbs light[4]. In the purple sulfur bacterium *Chromatium*, $P870^+$ extracts an electron from a *c*-type cytochrome, returning to the reduced state with a half-time of 2 μsec (ref. 3). The cyclic return of an electron from the primary electron acceptor back to the cytochrome requires several msec or longer.

The identity of the primary electron acceptor is unknown, and this problem presents a major challenge to our understanding. By lowering the redox potential chemically, one can reduce the electron acceptor in the dark, blocking P870 photooxidation[5,6]. With P870 preparations from *Rhodopseudomonas spheroides*, a chemical reduction of this type follows a one-electron titration with a midpoint potential of

Abbreviation: PMS, *N*-methylphenazonium methosulfate.

−50 mV (ref. 5). In a complementary experiment, NICOLSON AND CLAYTON[7] found that dyes with E_0' values more positive than −50 mV will reoxidize the reduced form of the acceptor. With *Rhodospirillum rubrum*, the chemical titration is closer to a 2-electron reaction with a midpoint potential of −20 mV (ref. 6).

No ESR signal from the reduced (or oxidized) acceptor has been found, perhaps because the signal is very broad, even at low temperatures, or perhaps because rapid electron pairing follows the primary reaction. Oxidized P870, on the other hand, does give a characteristic ESR signal, which matches that of known porphyrin radical cations[8–10].

The use of two short actinic flashes offers a different attack on the nature of the primary electron acceptor. If a second flash follows the first one sufficiently closely, it might find the electron acceptor still in the reduced form and unable to participate in further photochemistry. By varying the time between the two flashes, one could then investigate the kinetics of reoxidation of the acceptor. As the present report describes, the reoxidation requires a time on the order of 60 μsec in chromatophores, and is sensitive to pH and temperature.

MATERIALS AND METHODS

Chromatium Strain D was grown photoautotrophically in the medium of MORITA *et al.*[11]. The temperature was 30°, and the culture period was 3 days. The preparation and storage of chromatophores followed earlier techniques[12].

For most of the experiments, the chromatophore suspension was placed in a 1-cm square cuvette with four clear sides. The top of the cuvette featured a gas-tight rubber septum. A stream of N_2, which had passed over hot copper to remove O_2, was introduced through syringe needles, and bubbled through the suspension for 5 or 10 min before an experiment. For temperature control (in the experiment of Fig. 9 only), the cuvette resided in a chamber within a copper bar, which was held inside a Dewar flask. At the top, the bar was clamped to one plate of a thermoelectric module (EG and G, Beford, Mass., Model H-9-65). The upper plate of the thermoelectric module was clamped to a copper block, through which water or air could run. Passage of a controlled current through the module allowed heating or cooling the cuvette. A thermistor at the cuvette indicated the temperature. In all other experiments, the cuvette temperature was approx. 22°.

Fig. 1 diagrams the spectrophotometer, which is similar to that of DEVAULT[13]. The measuring lamp for P870 measurements was a xenon flash lamp (Type FX-76, EG and G), which discharged an energy of about 10 J. Pulse shaping networks with 3–5 inductance–capacitance sections provided a plateau of 20–60 μsec in the lamp output. A monochromator between the cuvette and the photomultiplier aided in the rejection of bacteriochlorophyll fluorescence and actinic light. Cut-off filters and interference filters generally supplemented the second monochromator. For cytochrome measurements, a 45-W tungsten–iodine lamp provided continuous measuring light, and filters replaced the second monochromator altogether.

The photomultiplier output entered an integrated circuit amplifier (Fairchild μA702) with an additional emitter follower at the output to decrease the output impedance. The amplifier acted as a current-to-voltage transresistor, with a low input

impedance. An offset current to the amplifier input balanced the d.c. signal from the photomultiplier.

Two Q-switched ruby lasers (Korad Corp., Santa Monica, Calif., Model K-1-QP) provided short actinic light pulses. Several different ruby rods were used, giving pulse lengths which varied between 14 and 40 nsec at half amplitude. The lasers were Q-switched with Korad Corp. pockels cells, which could be triggered on external voltage signals. A bank of monostable multivibrators provided the delays and trigger pulses which were necessary in order to synchronize the pockels cell triggers and the pump lamp flashes with opening the oscilloscope camera shutter and triggering the measuring lamp and the oscilloscope sweep.

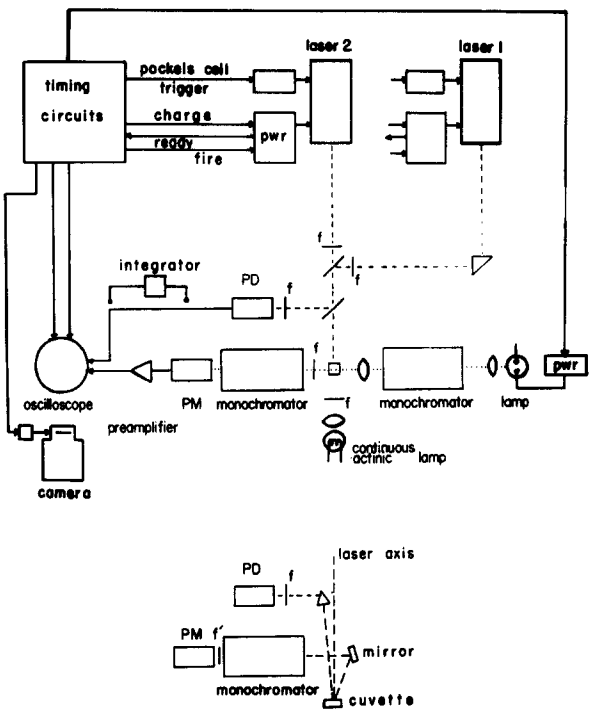

Fig. 1. Diagram of the single-beam kinetic spectrophotometer. Solid lines indicate paths of electrical signals; dotted or dashed lines, optical paths. Abbreviations: f, optical filter; PD, photodiode; PM, photomultiplier; pwr, power supply. Other symbols indicate prisms, mirrors, and beam splitters. The monochromators are Bausch and Lomb, No. 33-86-25, with appropriate gratings. The photomultipliers are RCA 7102 for P870 measurements and EMI 9558QA for cytochrome. See the text for other details. The lower part of the figure shows an alternative optical arrangement, for measuring low-angle fluorescence from a 0.1-cm cuvette.

One strength of the pockels cell Q-switches lies in the simplicity of firing the two lasers with precise timing. Following the trigger pulse, the jitter in the laser flash is on the order of 10 nsec. There is one major drawback to the pockels cell, however, and this is the radiofrequency noise which it emits when it fires. One must exercise care in the construction of the measuring apparatus, so as to minimize the pickup of this noise. The present measuring apparatus includes an elaborate system of double

shielding. Efforts at improving the shielding and grounding of the pockels cell itself were less rewarding.

Interference reflecting filters allowed attenuation of the laser beams. A fast photodiode (Type F4000, ITT Industrial Laboratories, Fort Wayne, Ind.) monitored a portion of the flash. Before reaching the photodiode, this portion of the beam passed through neutral density filters, an aperture which was the same size as the active part of the cuvette, and several ground glass diffusing plates. An integrator, in which a capacitor charged through several fast signal diodes in parallel, could be used with the photodiode to monitor the total laser energy. The photodiode and integrator were calibrated by comparison with a ballistic thermopile (Model 100, TRG Co., Melville, N.Y.) placed in the cuvette position. The oscilloscope was a dual beam instrument (Tektronix 556) which allowed simultaneous measurement of the photodiode and photomultiplier signals.

For fluorescence measurements, the face of a 1-mm cuvette was adjusted so that its normal made a small angle to the laser axis (lower portion of Fig. 1). The incident laser flash energy was measured by directing the specular reflection from the cuvette to the photodiode. Fluorescence emerging from the front face of the cuvette entered a monochromator *via* a mirror and then proceeded through cut-off filters (Wratten 87 and 88A) to the photomultiplier. The monochromator was set at 920 nm, with a band pass of 5 nm. Controls with white paper or water in the cuvette ruled out any direct effect of the laser flash on the photomultiplier. By adapting the monochromator and filters deliberately to allow laser light to reach the photomultiplier, it was shown that a comparatively strong flash had no significant effect on the instrumental response to a weaker flash several μsec later.

Either cuvette arrangement allowed continuous actinic illumination of the sample with a 75-W tungsten–iodine lamp and a Corning 2600 or Schott RG-N9 near-infrared filters. An FX-76 Xe lamp, with no added inductance, replaced the continuous lamp for experiments with three actinic flashes. The flash lamp had a pulse width of about 8 μsec at half amplitude.

RESULTS AND DISCUSSION

Fig. 2 shows measurements of P870 oxidation in *Chromatium* chromatophores. The laser flash causes a rapid P870 oxidation, which appears as an upward deflection in the oscilloscope trace. A reduction follows with a half-time of 2 μsec, as cytochrome C422 relinquishes an electron. A second flash 4–6 μsec after the first has quite a different effect. The oscilloscope trace shows only a brief artifact, which is due to fluorescence and scattered laser light reaching the photomultiplier. Essentially no P870 oxidation occurs on the second flash, even though most of the $P870^+$ which formed on the first flash has already returned to the reduced state. Some condition other than the oxidation state of P870 prevents P870 oxidation on the second flash.

Another way to determine the capacity for photochemistry at the time of the second flash is to measure fluorescence from the bulk bacteriochlorophyll. VREDENBERG AND DUYSENS[14] have shown that P870 oxidation competes directly with fluorescence, so that when P870 oxidation can occur the fluorescence yield is low. The detailed kinetics of this competition may vary, depending on whether energy transfer can occur from one photosynthetic unit to another[15], but oxidation of all of the P870

by continuous actinic light generally causes an increase in the fluorescence yield by a factor of 2–4.

Fig. 3 presents measurements of the bacteriochlorophyll fluorescence which results from a Q-switched laser flash, and Fig. 4 shows the results of a series of similar

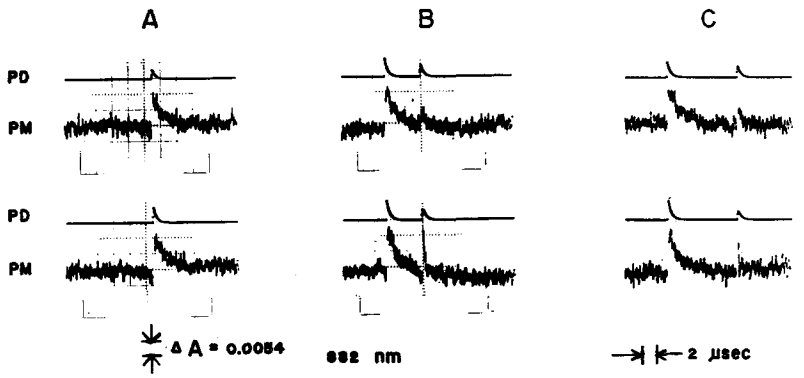

Fig. 2. P870 oxidation in *Chromatium* chromatophores. The figure shows 6 experiments, composed of 3 pairs. In each experiment, the trace marked PD shows the integrated photodiode record of the laser timing and energy. The trace marked PM shows the photomultiplier record of the optical transmission at 882 nm. An upward deflection means more light reaching the detector, in both cases. In a pair of experiments (A) there was only one actinic flash, from the same laser as generated the second flashes in B and C. In B, a second flash followed the first by 4.2 μsec; in C, it followed by 8.4 μsec. The flashes were of saturating intensity (several nEinsteins·cm^{-2} absorbed). 0.1 M glycylglycine buffer (pH 7.4); 40 μM PMS; 1-cm light path. (10-23-68.)

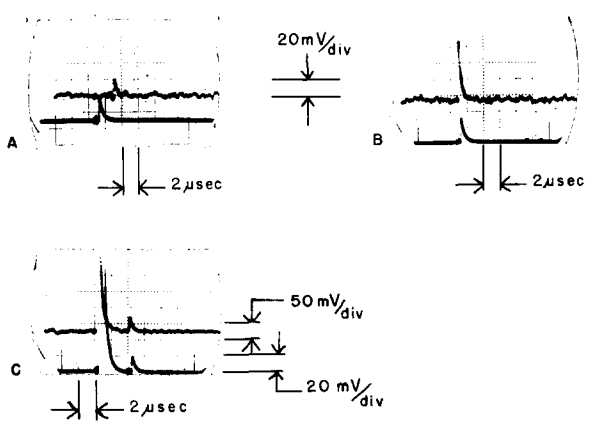

Fig. 3. Fluorescence measurements. The figure shows 3 experiments with weak test flashes. In each experiment, the lower trace shows the integrated photodiode record of the laser timing and energy. A 20-mV deflection on this trace represents an incident intensity of 0.17 nEinstein·cm^{-2}. The upper trace, with a higher noise level, shows the photomultiplier record of the bacteriochlorophyll fluorescence. The vertical scale (mV) is arbitrary. The triggering of the two traces was offset slightly in Expt. A. In A, the chromatophores were in darkness before the test flash. In B, they were illuminated by continuous near-infrared light (Corning 2600 filter), with an incident intensity of 1200 nEinsteins·cm^{-2}·sec^{-1}. In C, a strong flash, which drove both traces off scale, preceded the test flash, but the sample was otherwise in darkness before the test flash. The incident intensity of the strong flash was approx. 7.7 nEinsteins·cm^{-2}. Note that the fluorescence scale in C is different from that in A and B. 1 μM PMS; 1 mM ascorbate, glycylglycine buffer (pH 7.4). (9-26-68.)

measurements. The ordinate in Fig. 4 gives the integrated fluorescence per flash, in arbitrary units; the abscissa gives the integrated energy of the flash. The slopes of the curves indicate the differential fluorescence yield. With a single flash, the slope is low for weak flashes and is about 4 times greater for strong flashes, which saturate the capacity for photochemistry.

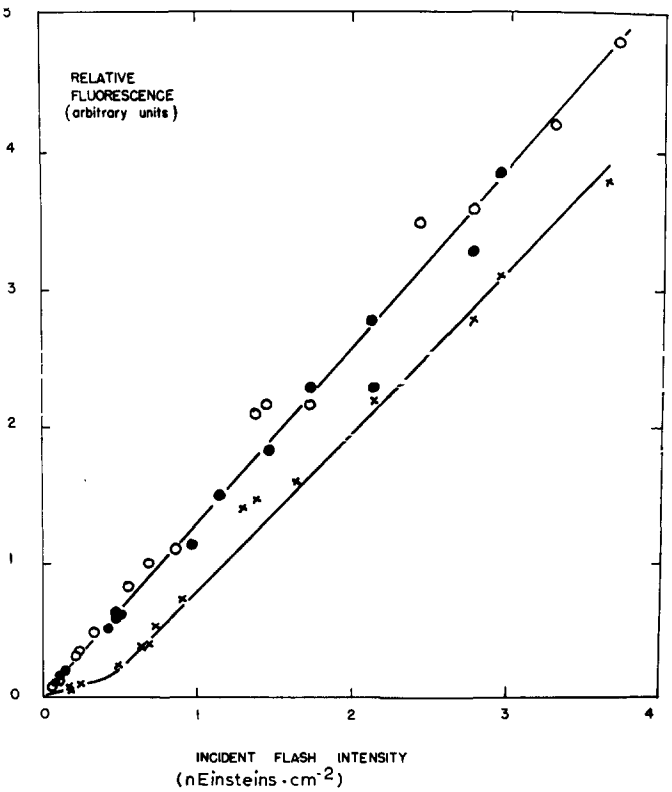

Fig. 4. Bacteriochlorophyll fluorescence resulting from a test flash, as a function of the intensity of the test flash. Conditions as in Fig. 3. ×—×, chromatophores in darkness before test flash, as in Fig. 3A; ○—○, chromatophores exposed to continuous near-infrared illumination, as in Fig. 3B; ●—●, chromatophores exposed to a strong flash 4 μsec before the test flash, as in Fig. 3C. (9-26-68.)

On the assumption that the intrinsic rate constants for deexcitation by fluorescence and by rationless (thermal) transitions are constant, the quantum yield of photochemistry in weak flashes is

$$1 - \frac{\phi_{DFW}}{\phi_{DFS}} \approx 0.75$$

where ϕ_{DFW} and ϕ_{DFS} are the differential fluorescence yields with weak and saturating flashes[16]. This is somewhat smaller than the quantum yield which one obtains by measuring P870 or cytochrome oxidation directly. The direct measurement requires an assumption of the differential extinction coefficient, $\Delta\varepsilon$, for oxidized-*minus*-reduced P870. REED AND CLAYTON[2] have measured $\Delta\varepsilon$ as 93 $mM^{-1} \cdot cm^{-1}$ at 865 nm (λ_{max})

for *Rps. spheroides* P870. Assuming that $\Delta\varepsilon = 93$ $mM^{-1} \cdot cm^{-1}$ at 882 nm (λ_{max} in *Chromatium*) gives a quantum yield of about 0.97 (ref. 3).

Fluorescence measurements would underestimate the quantum yield of chemistry if the trapping rate by $P870^+$, which may be assumed to be photochemically inactive, would not be negligible. In the case of green plant Photosystem I, the chlorophyll fluorescence yield is completely independent of the oxidation state of P700 (ref. 17), as though an exciton reaching $P700^+$ has essentially no chance of returning to the bulk chlorophyll. To account for this phenomenon, DUYSENS[17] postulates the existence of an additional chlorophyll form between the bulk chlorophyll *a* and P700, but this postulate may be unnecessary. $P870^+$ has an absorption band at 1.25 μ, along with weaker bands at approx. 0.98 and 1.16 μ (ref. 2). Electronic energy might undergo degradation to heat by way of these bands.

Returning to Figs. 3 and 4, one can saturate photochemistry in the chromatophores by irradiating with strong continuous light. The fluorescence yield is then high, even for weak test flashes. One obtains a similar result by the addition of $Na_2S_2O_4$ (not shown in Figs. 3 and 4). Presumably by reducing the primary electron acceptor, $Na_2S_2O_4$ prevents P870 photooxidation[3].

If, instead of irradiating the chromatophores continuously, one exposes them to a strong flash 4 μsec before the test flash, then the fluorescence yield from the test flash is high, even for weak test flashes (Figs. 3 and 4). This result supports that of Fig. 2. 4 μsec after the first flash, P870 oxidation does not occur and an increased portion of the excitation energy escapes as fluorescence. There is no indication that the absorbed quanta turn to an alternative photochemical reaction if P870 oxidation is blocked.

To determine the kinetics with which the capacity for photochemistry reappears after a flash, it is desirable to have still a third method for measuring photochemistry. On the one hand, direct P870 measurements are difficult because of the fluorescence artifact, the pulsed measuring light which is required, and the low signal-to-noise ratio. On the other hand, the possibility of energy transfer among different photosynthetic units complicates the relationship between bacteriochlorophyll fluorescence and P870 oxidation. Fortunately, P870 reduction is tightly coupled to cytochrome C422 oxidation, which one can measure with greater precision. At least, the coupling to cytochrome C422 is tight after a single flash, in the presence of a small amount of *N*-methylphenazonium methosulfate (PMS)[3].

Fig. 5 shows that a second flash 500 μsec after the first causes the oxidation of additional cytochrome *c*. If one places the two flashes closer together, the effect of the second flash diminishes. When the two flashes are less than 3 μsec apart, their total effect is the same as that of the first flash alone.

Three types of measurements thus indicate that the photochemical system enters a refractory state after a short flash. It is still in this state at a time when most of the P870 has returned to the reduced state. The simplest interpretation is that the primary electron acceptor, X, is still in possession of the electron which it took from P870, and that no photochemistry can occur until X^- loses this electron. This interpretation immediately restricts one's concept of X. The primary electron acceptor must interact stoichiometrically with P870, and it must be capable of accepting only one electron from P870. This does not exclude the possibility that X^- rapidly takes on a second electron from another source.

Several authors[19–22] give a similar interpretation to changes in fluorescence yield from System II of green plants. In this case also, FORBUSH AND KOK[19] find that a single 20-μsec flash is sufficient to raise the fluorescence yield to its maximal value.

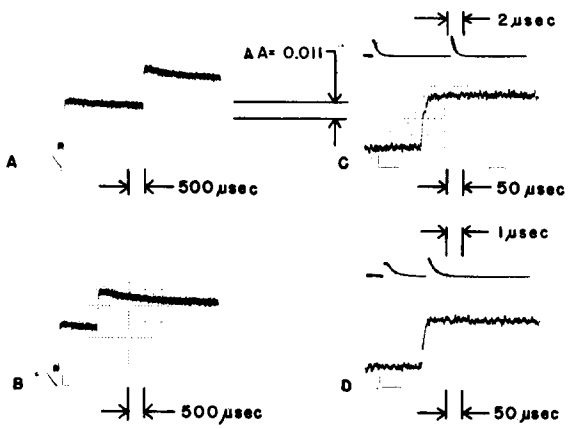

Fig. 5. Cytochrome oxidation resulting from two actinic flashes. A. Two flashes 2.4 msec apart. The trace is the photomultiplier record of the optical transmission at 422.5 nm. B. Two flashes 1.2 msec apart. C. Two flashes 9.6 μsec apart; the upper trace is the integrated photodiode record of the flash timing and energy; the lower trace, at a different sweep rate, is the photomultiplier record of the optical transmission at 422.5 nm. The effects of the two flashes are almost superimposed on the photomultiplier trace. D. Two flashes 2.6 μsec apart. The traces are as in C, except that the sweep rate of the photodiode trace is higher. In D, the effects of the two flashes are superimposed on the photomultiplier trace, and the total effect is the same as that of the first flash alone in A or B. Bacteriochlorophyll, 50 μM; PMS, 4 μM; buffer: 0.1 M phosphate, 0.1 M Tris, 0.1 M acetate (potassium salts), pH 7.5. (2-28-69.) The photographs of the photodiode responses are retraced with a pen in some places, for improved reproduction in the figure.

There is at least one other possible interpretation of the refractory state which persists after a flash in *Chromatium*. *Chromatium* may contain two different types of photochemical systems[23]. If this is the case, only one type may be capable of operating on a brief actinic flash. Without specifying why the second system is inactive, one could imagine that the refractory period is required for its activation. It appears safe to reject this interpretation, however, at least for *Chromatium* chromatophores. The following report[24] presents evidence that identical cytochromes participate on the first and the second flashes, that the mechanisms of their oxidation are identical, and that the first flash causes the oxidation of all of the P870 which is available in the chromatophores. Apparently each P870 can oxidize two cytochrome molecules.

If the refractory period reflects the accumulation of X^-, one can now investigate the kinetics with which X^- returns to the oxidized state, X. Fig. 6 shows the cytochrome oxidation which occurs on the second flash, as a function of the time between the two flashes. If the two flashes are less than 3 μsec apart at pH 7, or 7 μsec at pH 8, the second flash has essentially no effect. The second flash has its maximum effect if the two are about 1 msec apart. It has half this effect at about 60 μsec at pH 7 or 80 μsec at pH 8. The kinetics are the same in the absence of PMS, which affects only the total amount of cytochrome C422 which is available for photooxidation[24].

These kinetics are not cleanly first order. They generally give a rough fit to

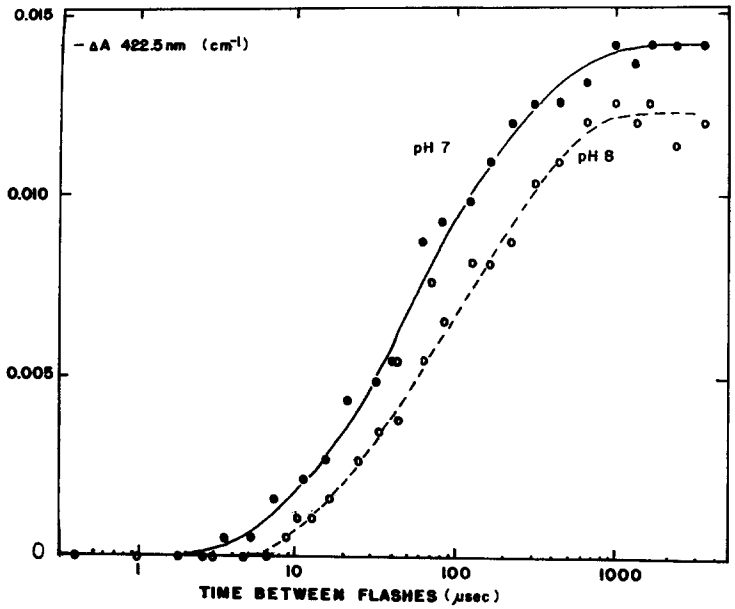

Fig. 6. Cytochrome oxidation caused by the second flash, as a function of the time between the two flashes. Conditions as in Fig. 5, except for the pH. (The buffer included the same components as in Fig. 5.) When the two flashes were more than about 20 μsec apart, the effects of both flashes were measured directly from photographs like those of Fig. 5. When the two flashes were closer together, the total effect of the two was measured, and the effect of the second flash was calculated by subtracting the effect of the first flash, which was assumed to be the same as it was for longer spacings. The abscissa scale is logarithmic. (11-9-68.)

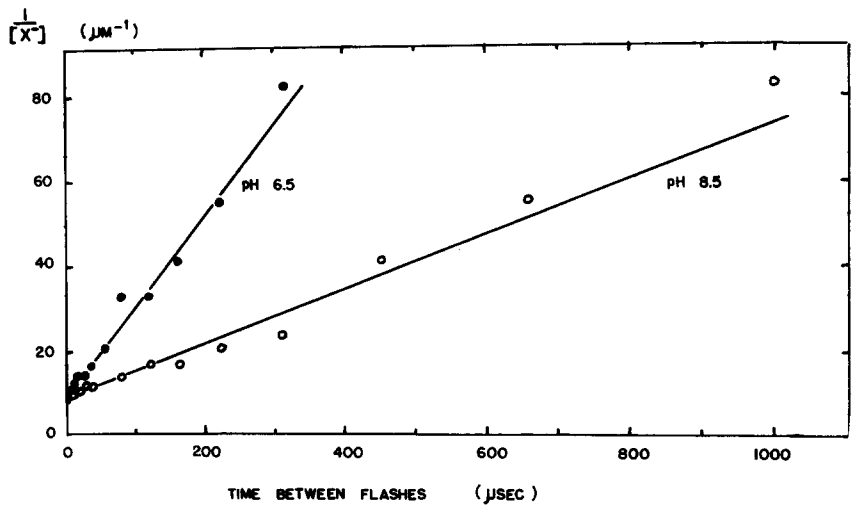

Fig. 7. Concentration of the reduced electron acceptor, as a function of the time after the first flash. This is a second order plot from data similar to those of Fig. 6. The ordinate plots the reciprocal of the concentration of X^-, the reduced primary electron acceptor. The X^- concentration is taken to be the difference between the maximum amount of cytochrome which the second flash can oxidize (if it comes a sufficiently long time after the first flash) *minus* the amount which it oxidizes at a given time. Absorbance changes like those of Fig. 6 were converted to concentration units on the assumption that $\Delta\varepsilon$ for cytochrome C422 oxidation is 90 $mM^{-1} \cdot cm^{-1}$ (see ref. 3). Conditions as in Figs. 5 and 6, except for the pH. (The buffer included the same components.) (11-11-68.)

a second order equation (Fig. 7). This rather surprising result suggests that two photosynthetic units interact, and that electron pairing occurs in this step. There are, however, difficulties in proving that X^- reoxidation is definitely second order in $[X^-]$. Plots of $1/[X^-]$, *versus* the time between the two flashes, have a large error for small $[X^-]$, so that they are satisfactory only over an 8-fold range of $1/[X^-]$. A combination of several first order terms could easily give the same fit in this plot, over such a small range of the reciprocal. If the reaction center population of the chromatophores is heterogeneous, the kinetics might reflect many different first order rate constants.

A subsequent report will describe similar kinetic measurements with *Chromatium* whole cells. The fit to a simple first order equation is no better with cells than it is with chromatophores, indicating that heterogeneity arising during cell disruption is not at fault. In fact, the X^- reoxidation in anaerobic whole cells is slower than it is in chromatophores. At pH 8, the half time in anaerobic cells is approx. 1 msec, compared to 80 μsec in chromatophores. This may be due to a more negative redox potential prevailing in whole cells, because aeration of the cells accelerates the reaction, decreasing the half-time to 200 μsec. CLAYTON[15] anticipated such a situation, in his discussion of bacteriochlorophyll fluorescence in whole cells.

A third flash can cause the oxidation of further cytochrome C422, but the kinetics of X^- reoxidation between the second and third flashes are more difficult to measure than are those between the first and second flashes. For these measurements, the two laser flashes were separated by 2–3 msec, and a Xe flash lamp provided the third flash. The maximum cytochrome oxidation by the third flash is comparatively small, because the first two flashes oxidize approx. 75 % of the cytochrome C422 which is available in the chromatophores. Nonetheless, X^- reoxidation appears to occur at approximately the same rate after the second flash as it does after the first. The third flash has half of its maximal effect if it follows the second by 60–80 μsec. With anaerobic whole cells, the situation is less clear, as a period of more than 1 sec is required before the third flash is fully effective.

If the refractory period after the second flash is the same as that after the first, electron accumulation at the secondary acceptor, Y, must not limit photochemistry in the chromatophores. This means, either that Y^- undergoes reoxidation in less than 60 μsec, or that Y provides a large sink for electrons. Ubiquinone could offer such a sink; its concentration exceeds that of P870 at least 4-fold. (This can be calculated from data in refs. 25 and 26.)

As Figs. 6 and 7 indicate, the X^- reoxidation rate is a function of pH. Fig. 8 shows the pH dependence in more detail. As a measure of the X^- reoxidation rate, the figure presents the reciprocal of the time ($\tau_{1/2}$) which must elapse between two flashes, before the second flash will cause half-maximal cytochrome oxidation. This measure would be perfectly satisfactory only if the kinetics were cleanly first order, which they are not. However, plots based on apparent second order rate constants give essentially the same picture. The rate increases with increasing $[H^+]$ over the pH range 9–5.5, suggesting that a proton participates in the reaction. The effect of pH on the kinetics may be somewhat more subtle than a stoichiometric entry of a proton, because the plot of log $(1/\tau_{1/2})$ *vs.* log $[H^+]$ has a slope of less than 1 (0.37 in Fig. 8).

REED *et al.*[5] found the redox potential of the primary electron acceptor to be essentially independent of pH. They measured the yield of P870 fluorescence in a

preparation from *Rps. spheroides*. The fluorescence increased upon the addition of reduced dyes, following a one-electron titration with a midpoint potential (−50 mV) which was independent of pH. If a proton participates in X^- reoxidation, the experiments of REED *et al.*[5] indicate that it must do so by affecting the secondary electron acceptor, Y, and not by reacting with X^-. This conclusion would be consistent with identifying Y as ubiquinone or a flavin, either of which would take on a proton as it underwent reduction.

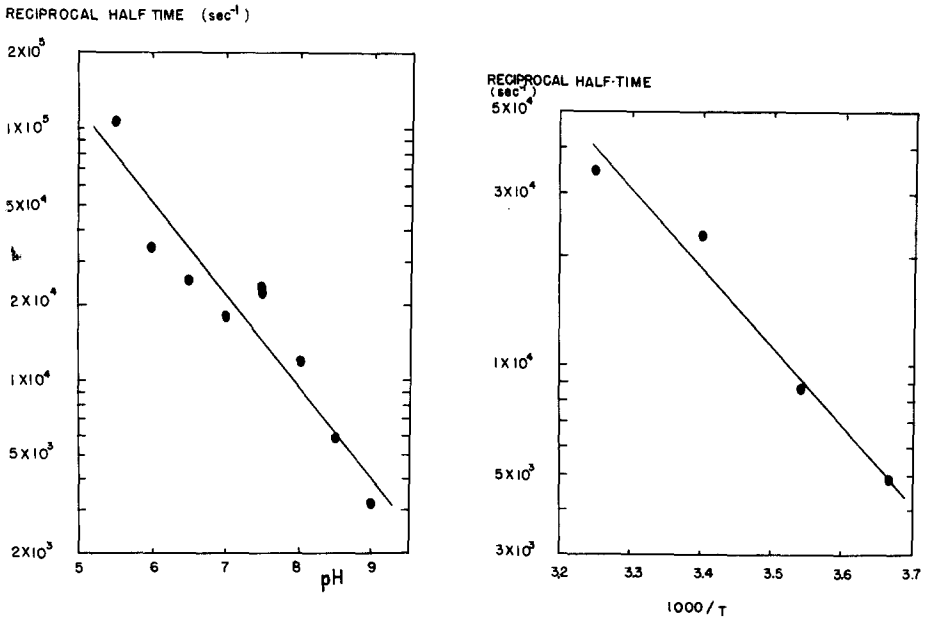

Fig. 8. Rate of reoxidation of the reduced electron acceptor, as a function of the pH. The ordinate gives the reciprocal of the time which must elapse after the first flash, before the second flash has half of its maximal effect. This time is determined from semilog plots like those of Fig. 6. Conditions as in Figs. 5 and 6. All buffers included the same components. The ionic strength was not constant over this pH range, but separate measurements showed that varying the ionic strength between 0.02 and 0.50 by the addition of NaCl to a sodium phosphate buffer at pH 6.8 had no significant effect on the X^- oxidation rate. (11-4-68.)

Fig. 9. Rate of reoxidation of the reduced electron acceptor, as a function of the reciprocal of the absolute temperature. The ordinate is similar to that of Fig. 8. Conditions as in Figs. 5 and 6. The data shown here are uncorrected for the change in the pH which occurs with temperature. The pH changed from 7.83 at 0.5° to 7.41 at 34.8°. (2-27-69.)

There is one difficulty in identifying Y as ubiquinone. KE[27] has found that a 275-nm absorbance decrease, which is widely attributed to ubiquinone reduction, can occur within 1 μsec after an actinic flash. This absorbance change cannot represent the primary electron acceptor, because it does not occur under mildly reducing conditions which still allow P870 oxidation[5,27]. If ubiquinone is a secondary acceptor, then X^- must reduce it in less than 1 μsec, but this is clearly inconsistent with the time course of Fig. 6. The simplest answer to this dilemma may be that the 275-nm absorbance change is due to something other than ubiquinone reduction, at least in part. To the arguments on this point which are already in the literature[28], one

may add the following considerations. First, as one lowers the redox potential, the dark reversal of the 275-nm absorbance decrease becomes faster[5]. This suggests that the light-induced absorbance decrease reflects an oxidation, not a reduction. Second, application of the differential extinction coefficient for ubiquinone reduction forces one to the disconcerting conclusion that two electrons reach ubiquinone for every electron which leaves P870 (ref. 29).

Fig. 9 shows the temperature dependence of the X^- reoxidation rate. The rate increases sharply with rising temperature. A plot of log $\tau_{1/2}^{-1}$ *vs.* $1/T$ fits a straight line with a slope of 2200°K. Again, a plot of apparent second order rate constants gives essentially the same picture. Calculation of the apparent activation energy, E_a, requires a correction for the change in the buffer pH with temperature. The buffer pH was measured over the temperature range of Fig. 9 and the straight line of Fig. 8 was used to make the correction. The corrected value of E_a is 8.3 kcal/mole.

P870 photooxidation still occurs at temperatures as low as 1°K (ref. 30). The temperature dependence of electron transfer between X^- and Y insures that this step does not occur at very low temperatures. In chromatophores of *Chromatium* and *Rsp. rubrum*, cytochrome oxidation also fails at low temperatures, yet the $P870^+$ returns to the reduced state in a dark reaction, with a half-time on the order of 20 msec. As the reactions of both the secondary electron acceptor (Y) and the secondary electron donor (cytochrome) are blocked, the dark reaction must be a reversal of the primary light reaction. The rate of this reaction actually increases with falling temperature[12], or at least remains constant between 1 and 77°K (ref. 8), suggesting that the electron tunnels through the energy barrier.

The identity of X remains a mystery, but the following conclusions may aid in future attacks on this problem. First, X appears to occur in 1:1 stoichiometry with P870, whereas Y may form a larger pool. Second, X^- reoxidation is not hopelessly fast. Strong, continuous actinic light should cause the accumulation of a significant portion of X^-. Third, the reoxidation rate is a function of pH and temperature, such that low temperature and high pH favor the accumulation of X^-. And finally, if X reduction is accompanied by an optical absorbance change or an ESR signal change, this signal should disappear after a flash with kinetics which match those of Fig. 6. No absorbance changes with these kinetics have been discovered as yet in *Chromatium* chromatophores. Earlier experiments[3,31] did suggest that a small amount of cytochrome reduction occurred with a half-time of 50 μsec after a flash, but this event was not seen in the present experiments. G. SEIBERT AND D. DEVAULT (personal communication) have found that the apparent 50-μsec reaction is an instrumental artifact.

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