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FLASH-INDUCED ABSORBANCE CHANGES IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES*

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SUMMARY

1. Following a 30 nsec flash from a ruby laser, P870 absorbance changes in *Rhodospirillum rubrum* chromatophores are complete in less than 1 μsec, even at 37 °K. The spectrum of absorbance changes is similar to that obtained with continuous illumination. At 37 °K, the principal absorption band of P870 is at 891 mμ, 27 mμ to the red of the room temperature position.

2. The quantum requirement for P870 bleaching is 3.6 quanta per molecule. The dependence on flash intensity is the same at 430 mμ and 792 mμ.

3. The absorbance changes at 430 mμ, 742 mμ and 870 mμ recover in the dark at the same rates. The recoveries involve 3 groups of steps. The steps with half-times in the range 10–200 msec are sensitive to heating and inhibitors of photosynthesis. They increase in rate with rising temperature.

4. In the presence of antimycin, oxidation of cytochrome *c* and reduction of cytochrome *b* follow a flash. The half-times are 10–20 msec. At approximately the same rate as cytochrome *c* is oxidized, about twice as much P870 recovers.

5. Reduced *N*-methylphenazonium methosulfate (PMS) accelerates the P870 recovery in a reaction which is first order in PMS and $P870^+$. The reaction is faster than the cytochrome *c* oxidation, and increases in rate with rising temperature. No simultaneous oxidation of PMS was detected.

6. At low temperatures, the P870 recovery is kinetically first order. Its rate increases with decreasing temperature, at least down to 29 °K.

7. Dithionite does not cause loss of the P870 absorbance changes, but causes a recovery with a half-time of several μsec. This recovery also occurs at low temperatures, decreasing in rate as the temperature is lowered.

INTRODUCTION

Illumination of chromatophores from photosynthetic bacteria causes absorbance changes which may be important for an understanding of bacterial photosyn-

Abbreviations: BChl, bacteriochlorophyll; FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; PMA, phenylmercuric actetate; PMS, *N*-methylphenazonium methosulfate; DCIP, 2,6-dichlorophenolindophenol; ESR, electron spin resonance.

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thesis[1-6,10]. Remarkably, these absorbance changes are reversible in the dark even at 1 °K[7,8]. There relevance to photosynthesis has been open to question, however, because they differ from the absorbance changes which illumination causes in intact cells, and because they have appeared to be insensitive to heat or inhibitors of photosynthesis.

In fresh cells of photosynthetic bacteria, light causes absorbance changes associated with cytochrome oxidation[9-12], whereas in chromatophores, cytochrome oxidation generally is not apparent except after special treatments. The major features of the absorbance changes found in *Rhodospirillum rubrum* chromatophores are[5,10,13]: *A* decreases at 870 mμ, 815 mμ, 605 mμ and 390 mμ and *A* increases at 792 mμ and 365 mμ, all of which have been attributed to bacteriochlorophyll (BChl), a broad *A* increase centering at 430 mμ, due to an unidentified material, and an *A* decrease at 270 mμ attributed to ubiquinone reduction[5]. None of these occurs in fresh whole cells exposed to continuous illumination of low intensity. In fact, the absorbance at 275 mμ changes in the opposite direction, as if ubiquinone becomes more oxidized[14]. If intact cells are treated with inhibitors such as hydroxylamine, illumination then causes absorbance changes similar to those seen in chromatophores[7].

In spite of these apparent difficulties, one currently popular view[2-4,7,15-17] is that the infrared absorbance changes in chromatophores indicate the photo-oxidation of a specialized BChl, designated "P870". An oxidation seems more likely than a reduction, because the addition of reductants hastens the dark reversal of the optical change, and because the addition of chemical oxidants can duplicate the effect of illumination[2,4,5,17]. The core of this theory is that oxidation of P870 is the first chemical reaction which follows the capture of light energy by BChl. In a second step, the oxidized P870 ($P870^+$) is reduced again by cytochrome.

A rival theory[11] is that the initial chemical reaction in bacterial photosynthesis is the oxidation of a cytochrome. In this theory, the P870 absorbance changes indicate a diversion of energy from its normal fate.

With the development of apparatus for measuring rapid absorbance changes after a flash from a ruby laser[18], DEVAULT has found that the 430 mμ *A* increase does occur in whole *R. rubrum* cells, but that it recovers after a flash with a half-time of 25 μsec (ref. 19). Cytochrome appears to be oxidized as the 430 mμ pigment recovers. The rapidity of the dark recovery may explain why a steady-state *A* change at 430 mμ is detectable in whole cells only at high light intensities.

In the present work, the laser apparatus was used to investigate the absorbance changes in *R. rubrum* chromatophores. Although the initial absorbance changes occur too rapidly for us to follow, the recoveries in the dark after a flash are measurable, and are sensitive to inhibitors of photosynthesis, to oxidants and reductants, and to temperature. Studies in the near infrared and visible regions of the spectrum will be presented here, and those in the ultraviolet in a future communication.

MATERIALS AND METHODS

R. rubrum was grown anaerobically in the light (50- to 100-ft candles) in 0.2 % Difco yeast extract *plus* 0.3 % Difco casaminacids in distilled water. The cells were harvested after 4 days of growth, washed with 0.1 M potassium glycylglycine (pH 7.4), resuspended in the same buffer (1 ml/g wet cells), and sonicated for 60 sec

with a Branson 20-kcycles sonifier. After approximately two-fold dilution with buffer, and removal of debris by 10 min of centrifugation at 12000 × *g*, the chromatophore fraction was collected by 90 min of centrifugation at 144000 × *g*. Without further washing, the pellet was resuspended in ethylene glycol–0.1 M glycylglycine (4:6, v/v) pH 7.4, (1 ml/g original wet cells), and stored at −4°. For absorbance measurements, portions were diluted with 0.1 M glycylglycine buffer (pH 7.4). BChl was measured in methanol extracts; ε was assumed to be 42 $mM^{-1} \cdot cm^{-1}$ at 775 mμ (ref. 20).

The samples were generally aerobic during the measurements. For some experiments, most of the O_2 was removed by bubbling the sample with N_2 and layering it with mineral oil. This treatment had no apparent effect on the absorbance changes or their dark recoveries.

The laser apparatus has been described by DEVAULT[18]. Briefly, a Q-switched ruby laser provides a 30-nsec flash of 6943 Å actinic light. Measuring light from a 30-W tungsten lamp passes through a monochromator, through the sample, and then to a photomultiplier. The RCA 7102 photomultiplier is used for studies in the infrared and EMI 9592 B and 9524 B tubes for the visible. Appropriate Corning and Wratten filters guard the photomultiplier from the laser flash. The photomultiplier output is amplified, balanced with a d.c. offset voltage, and displayed on an oscilloscope.

Attenuating lenses and neutral density filters allowed control of the intensity of the flash and the measuring light. The flash intensity was measured with a TRG bolometer, and the measuring beam intensity with a Reeder thermopile. The output from both instruments was amplified with a Kiethley millimicrovoltmeter.

The cuvette, which had a 1.6-mm light path, was made with plexiglass windows mounted on an aluminum fin that could be immersed in a dewar of liquid N_2 or dry ice–ethanol. Refrigerant from a thermostated bath could be circulated through copper tubing on the fin for studies in the temperature range −10° to +25°. To reach 29 °K, we used an Air Products Corp. two-stage (N_2 and H_2) Joule–Thomson cryostat. The low temperature cuvette had sapphire windows for high thermal conductivity. It was screwed to the end of the cryogenic probe, surrounded by a heat shield at liquid N_2 temperature, and housed in a chamber which was evacuated by a Varian Vac-ion pump. To measure low temperatures, the output of a gold–cobalt *versus* "normal silver" thermocouple was amplified with a Millivac voltmeter. The voltage *versus* temperature curve used was taken from the data of POWELL, BUNCH AND CORRUCCINI[21].

RESULTS

General description of the absorbance changes

Absorbance changes in *R. rubrum* chromatophores caused by the laser flash are essentially identical to those caused by continuous illumination. Other workers have described these previously[5,6,13]. Fig. 1 gives portions of the low temperature difference spectrum, which has not been described in detail before. Because the 430 mμ band is notably broad at room temperature, it is of interest that it splits into two maxima in frozen samples. The 390 mμ band behaves similarly.

At 763 mμ, the shoulder which appears at room temperature stands out as

a sharp band at low temperatures. The 792 mμ and 815 mμ bands sharpen greatly at low temperatures and move together; the isosbestic point does not shift. As VREDENBERG AND DUYSENS[22] have noted for whole cells, the P870 band shifts dramatically to the red at low temperatures. At 34 °K the maximum is at 891 mμ, shifted 27 mμ (0.09 eV) from the room temperature position. The 360 mμ band shifts slightly to the red (4 mμ = 0.04 eV at 80 °K).

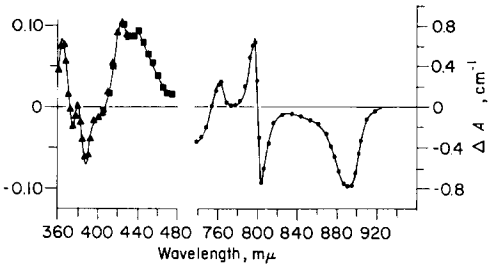

Fig. 1. Absorbance changes caused by a laser flash in frozen suspensions of *R. rubrum* chromatophores. Laser flash intensity: $8.7 \cdot 10^{-9}$ Einstein/cm². ●—●, 34 °K, 68 μM BChl; ■—■, 37 °K, 82 μM BChl; ▲—▲, 85 °K, 81 μM BChl. These were three different experiments. The experiment at 85 °K gave smaller *A* changes than the other two did, because the chromatophores were forced to the edges of the cuvette as the sample froze. The *A* changes in this experiment were multiplied by 4 to normalize them with those of the 37° experiment at wavelengths above 400 mμ.

The absorption spectrum of the bulk BChl (B890) also shifts to the red with decreasing temperature, from 878 mμ at 295 °K to 891 mμ at 34 °K. This shift is smaller than that of P870, so that at 34 °K the two absorption bands are superimposed. CLAYTON AND ARNOLD[23] have presented absorption spectra of dried films of *Rhodopseudomonas spheroides* chromatophores in which a shoulder on the red side of the B890 band increases to a major peak as the temperature is lowered. According to BRODY AND LINSCHITZ[24], the BChl fluorescence in *R. rubrum* also shifts from 900 mμ to 920 mμ between 295 °K and 77 °K. These changes in the B890 and P870 absorption bands are not due simply to freezing or to changes in the ice crystal structure which occur near 240 °K because spectra obtained at 203 °K and 77 °K are intermediate between those obtained at 295 °K and 34 °K.

The magnitude of the initial *A* changes was insensitive to the addition of inhibitors and to 65° heating. Heating the aerobic sample for 10 min at 80° destroyed the absorbance changes, and the addition of PMS and ascorbate could not restore them. CLAYTON[2,4], and KUNTZ, LOACH AND CALVIN[5] have noted that the steady-state absorbance changes are lost if one treats chromatophores with $K_3Fe(CN)_6$, and this proved true for the flash-induced changes as well. Absorbance changes in frozen samples (at 77 °K) were lost along with those at room temperature by prior treatment with the oxidant. Treatment with $Na_2S_2O_4$ did not cause loss of the optical changes, contrary to the results of LOACH[25], and KUNTZ, LOACH AND CALVIN[5]. This point is developed more fully below. As MAYNE AND CLAYTON[8] have reported for steady-state absorbance changes in *Rps. spheroides* chromatophores, the flash-induced changes did not occur at low temperatures if the chromatophore sample was illuminated with red light while it was being cooled.

VREDENBERG[26] and OLSON AND KOK[27], working with whole cells, reported that the steady-state absorbance change at 430 mμ saturates at lower actinic light in-

tensity than does that at 870 mμ. This may be due to an effect of the measuring light on the recovery rates (see below). In chromatophores, the initial absorbance changes have the same dependence on the laser flash intensity (Fig. 2).

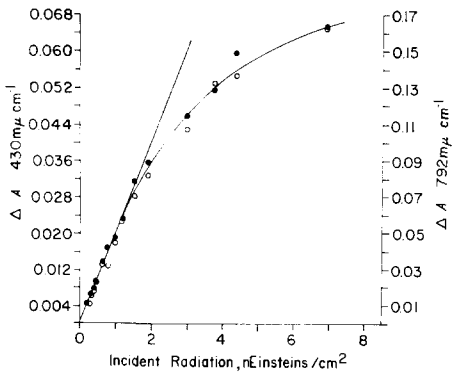

Fig. 2. Dependence of the absorbance changes on the laser flash intensity. ●—●, 430 mμ; O--O, 792 mμ. 140 μM BChl. 295° K.

The data of Fig. 2 allow an estimate of the quantum efficiency of the absorbance change if we used CLAYTON's[37] value of $\Delta\varepsilon$ for bleaching of P870, 113 $mM^{-1} \cdot cm^{-1}$. At room temperature, the flash-induced A increase at 792 mμ is about 2/3 as large as the A decrease at 864 mμ. Assuming that $\Delta\varepsilon$ at 792 mμ is 75 $mM^{-1} \cdot cm^{-1}$, the initial slope in Fig. 3 is 0.108 nmoles/n Einstein. The sample absorbed 39% of the 6943 Å incident light, according to measurements with a Zeiss spectrophotometer equipped with an integrating sphere. This gives a quantum requirement of 3.6 quanta per molecule of P870 bleached. Using a different method, CLAYTON[2] obtained quantum

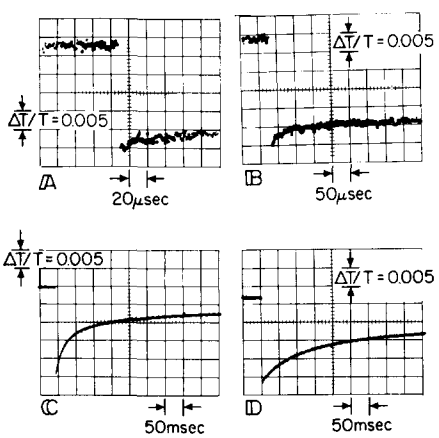

Fig. 3. Recovery of the absorbance changes in the dark, following a flash. Note that the oscilloscope sweep rate varies. A, 440 mμ, 140 μM BChl. B, same as A, except 431 mμ and slower sweep. C, 430mμ, 152 μM BChl. D, same as C, except after the chromatophores had been heated for 10 min at 60°. All traces: 295 °K. A downward deflection is an A increase. The upper and lower parts of the figure come from different experiments with different chromatophore preparations.

requirements of 2.3 to 6 quanta for *Rps. spheroides* and *Chromatium* chromatophores, and 7.0 for *R. rubrum**.

Rate of the initial absorbance changes

With the laser apparatus, one can see that the initial optical changes are complete in less than 1 μsec, which is approximately the response time of the apparatus (Figs. 3A and 16A). KUNTZ, LOACH AND CALVIN[5], using repetitive flashes, determined a first order rate constant of 16.3 sec^{-1} (half-time = 42.5 msec) for the A increase at 433 mμ, but their low actinic light intensity probably limited this rate. Even at 37 °K, the rate was too high for us to measure. At 870 mμ we were unable to make measurements sooner than about 10 μsec after the flash because of a brief, strong pulse from the photomultiplier. This was partly an artifact due to laser light reaching the tube and partly a response to fluorescence and delayed emission. At other wavelengths, including 390 mμ, 430 mμ, 605 mμ, and 792 mμ, we could measure within 1 or 2 μsec of the flash, and the absorbance changes were always complete by this time.

Dark recovery of the absorbance changes at room temperature

KUNTZ, LOACH AND CALVIN[5] measured the recovery rates after longer periods of illumination and determined that the recovery at 792 mμ was faster than that at 430 mμ, and the recovery at 865 mμ was faster still. They concluded that at least 3 different pigments caused the absorbance changes. OLSON AND KOK[27] obtained similar results. CLAYTON[2], however, reported that the kinetics at 430 mμ were identical with those in the infrared. The results of KUNTZ, LOACH AND CALVIN[5] and OLSON AND KOK[27] probably were due to an effect of the measuring light on the absorbance changes. Fig. 4 shows that decreasing the intensity of the measuring beam markedly affects the appearance of the recovery at 861 mμ. Similar attenuation of the 430 mμ measuring light had no significant effect on the recovery kinetics. When the 861 mμ beam was attenuated by a factor of 10, the kinetics at the two wavelengths become indistinguishable.

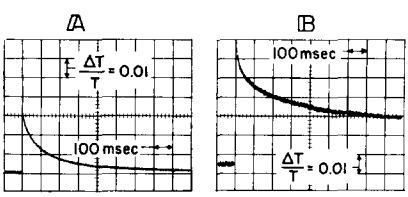

Fig. 4. Recovery kinetics at 861 mμ as observed with two different measuring light intensities. Both traces: 295 °K, 51 μM BChl, upward deflection is an A decrease, A, measuring beam unattenuated. The light striking the sample cuvette was $5.4 \cdot 10^{-11}$ Einstein/sec·cm². B, measuring beam attenuated 10-fold with a neutral density filter. The anode to cathode voltage of the photomultiplier was adjusted to give the same anode current in the two experiments.

Several factors may contribute to the difference between the two wavelengths. First, the output of the lamp and monochromator is about 10 times greater (in quanta/sec) at 861 mμ than at 430 mμ. With our lamp, the intensities are $5.4 \cdot 10^{-11}$

* The quantum requirements given in ref. 2 have been multiplied by a factor of 1.55. The most recent estimate of $\Delta\varepsilon$ for P870 bleaching is 113 $mM^{-1} \cdot cm^{-1}$, rather than 73 $mM^{-1} \cdot cm^{-1}$ as given in ref. 2.

Einstein/sec·cm^2 at 861 mμ and 5.1·10^{-12} Einstein/sec·cm^2 at 430 mμ. Also, the chromatophores absorb more of the 861 mμ light, and use the absorbed light more efficiently.

The continuous 861 mμ measuring light seems to bleach a portion of the BChl which has a long recovery time. The laser flash then bleaches an additional fraction of the BChl, a fraction which recovers rapidly enough so that the steady measuring light does not bleach it extensively. At 430 mμ the measuring light is too weak to have much effect, and the laser flash bleaches both fractions of the BChl*.

At room temperature, 5 to 20% of the absorbance change recovers with a half-time of approx. 20 μsec (Fig. 3B). This recovery step is insensitive to antimycin, HOQNO, and PMA, but it is destroyed by heating the chromatophores for 10 min at 65°. As the recovery causes an A decrease at 418 mμ, it is difficult to determine whether there is an additional fast A decrease at this wavelength indicating simultaneous cytochrome oxidation. The recovery rate is similar to that at which complete recovery occurs in whole cells[19], but much higher than the rate at which cytochrome c is oxidized in chromatophores which have been treated with antimycin (see below). The magnitude and especially the rate of the fast recovery step decrease as the chromatophores are stored in 40% ethylene glycol at −4°. The experiment of Fig. 3B illustrates the recovery in chromatophores which had been stored for 3 days. One week later, the half-time of the fast recovery had increased to 200 μsec.

We shall designate the 20-μsec recovery step as "type A". The remainder of the recovery separates into two groups of much slower steps, with half-times in the

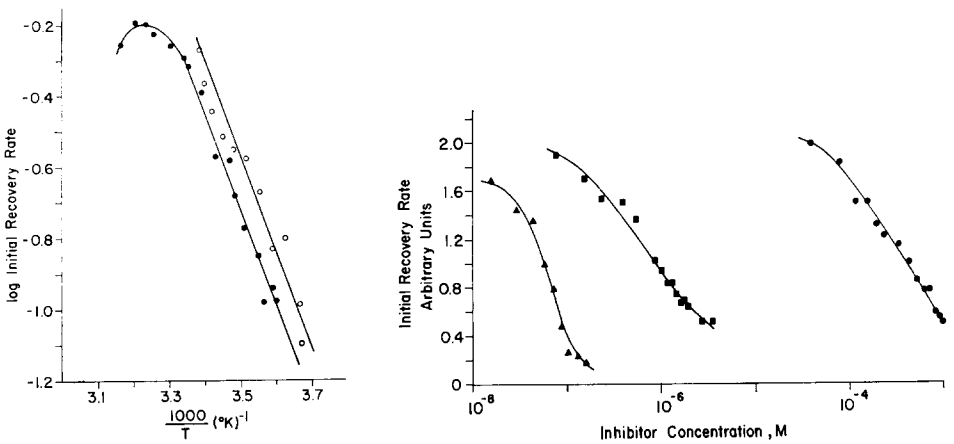

Fig. 5. Dependence of the B recovery on temperature. The log of the recovery rate is plotted against T^{-1}. The "rate" is the initial slope of the recovery, in arbitrary units. ●—●, 430 mμ, 152 μM BChl. ○—○, 792 mμ, 97 μM BChl. These were separate experiments, with two different chromatophore preparations. 295 °K.

Fig. 6. Sensitivity of the B recovery to inhibitors. 51 μM BChl, 295 °K. All measurements at 792 mμ. ▲—▲, antimycin A; ■—■, HOQNO; ●—●, PMA. The initial slope of the recovery, in arbitrary units, is plotted against the log of the inhibitor concentration.

* RUBY, KUNTZ AND CALVIN[36] found that the decay kinetics of the light-induced electron spin resonance (ESR) signal in *R. rubrum* chromatophores matched those of the light-induced 430 mμ A increase, but not those of the A changes in the infrared. They concluded that the ESR signal was not due to oxidized BChl (870$^+$). This conclusion is not essential if their infrared absorbance kinetics were distorted by the measuring light.

range 10 to 200 msec in the first group ("type B") and 1 to 4 sec in the second ("type C"). Figs. 3C and 4 show the B and C type recoveries. We could not describe the kinetics adequately with two or three first order terms although other workers have used this treatment for the recovery after continuous illumination[5,8].

Fig. 5 shows that the type B recovery steps become more rapid with rising temperature. Because the recovery rates do not resolve simply into a small number of terms, we can give only a rough description of the temperature dependence. The rate constants in Fig. 5 are the initial slopes of the recovery. These slopes fit an Arrhenius plot with $E_a = 5.7$ kcal/mole below 300 °K.

Heating the aerobic chromatophores for 10 min at 60° inhibits the type B recovery (Fig. 3D), but has little effect on the type C recovery. NOZAKI, TAGAWA AND ARNON[28] have shown that this treatment damages photophosphorylation, probably by causing the oxidation of an electron carrier.

Antimycin A, HOQNO, and PMA inhibit the type B recovery steps (Fig. 6). They appear to have little or no effect on the types A and C recoveries. While this manuscript was in preparation, GELLER[29] reported similar effects of antimycin and HOQNO. Several other inhibitors and uncouplers (33 μM FCCP, gramicidin (2 μg/ml), valinomycin (0.11 μg/ml), and 300 μM quinacrine) had no effect in our system, but GELLER[29] found uncouplers to accelerate the recovery. According to an earlier report of GELLER AND LIPMANN[1], and in our experience, antimycin, HOANO, and PMA have little effect on the steady-state absorbance changes or on the dark recovery after continuous illumination.

In the presence of antimycin, about 6% of the initial absorbance change recovers with a half-time of approx. 10 msec (Fig. 7A). This recovery can be seen in any of the absorption bands, but between 400 mμ and 440 mμ and between 540 mμ

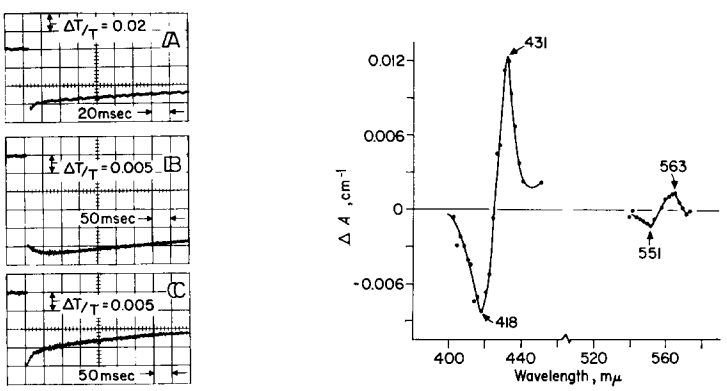

Fig. 7. Reactions following a flash in the presence of antimycin. 295 °K, 0.91 μM antimycin. A, 792 mμ, 115 μM BChl; B, 431 mμ, 122 μM BChl; C, 418 mμ, 122 μM BChl. Note that the oscilloscope sweep rate was greater in A than in B and C. Downward deflection is an A increase. The recovery in A was 12% of the initial change; generally, it was smaller, averaging about 6%.

Fig. 8. Spectrum of secondary absorbance changes following a flash in the presence of 0.91 μM antimycin. 122 μM BChl. In photographs like those of Fig. 7, the linear slower portions of the recovery were extrapolated to the time of the flash. The absorbance difference was measured between the extrapolated values and the actual value of the absorbance immediately after the A change on the assumption that a 6% recovery occurred at all wavelengths. This correction had little effect on the shape of the spectrum, but it did tend to bring the spectrum closer to the baseline at either end.

and 570 mμ additional optical changes are superimposed on it. Figs. 7B and C show these at 431 mμ and 418 mμ. At 431 mμ, an additional A increase follows the flash; at 418 mμ, and A decrease occurs. The latter (half-time 10 msec) appears somewhat faster than the former (half-time 18 msec), but this difference is only marginally significant because the small absorbance changes must be corrected for recovery of the principal absorbance change.

Fig. 8 shows a plot of the additional absorbance changes at different wavelengths. The difference spectrum is similar to one which NISHIMURA[30] obtained upon illuminating *R. rubrum* whole cells after treatment with HOQNO or antimycin. NISHIMURA's interpretation[30], which seems equally valid here, was that light causes reduction of cytochrome *b* ($\lambda\lambda_{max}$ 431 mμ, 563 mμ) and oxidation of cytochrome *c* ($\lambda\lambda_{max}$ 418 mμ, 551 mμ). Antimycin apparently blocks electron transport between the two cytochromes, as it does in mitochondria.

In the experiment of Fig. 8, 84 nM cytochrome *b* was reduced and 75 nM cytochrome *c* was oxidized, assuming $\Delta\varepsilon$ for reduction is $+17$ $mM^{-1}\cdot cm^{-1}$ in the α bands of both cytochromes. These amounts were quite small (only about 1 cytochrome per 1000 BChl). If $\Delta\varepsilon$ at 792 mμ is 75 $mM^{-1}\cdot cm^{-1}$ for P870 oxidation, 2.42 μM P870 was oxidized in Fig. 8a. A recovery of 6 % in the presence of antimycin is a restoration of 148 nM P870, or about twice the amount of cytochrome *c* which becomes oxidized. This discrepancy is of questionable significance because the absorbance coefficients are uncertain.

Recovery in the presence of reduced PMS

Rapid recovery of the absorbance changes occurs even in the presence of antimycin, HOQNO, or PMA, if PMS and a reductant are added. If the inhibitor is antimycin or HOQNO, either succinate or ascorbate can serve as the reductant. In the case of PMA, ascorbate, but not succinate, is a suitable reductant. Apparently, PMA, but not antimycin or HOQNO, blocks the enzymatic reduction of PMS by succinate. The addition of succinate or ascorbate alone, without PMS, has little or no effect on the recovery, either in the presence or in the absence of the inhibitors. DCIP behaves similarly to PMS, except that the dependence on the addition of a reductant is not quite as strict, and the maximum recovery rates attained are not as great.

GELLER AND LIPMANN[1] and BALES AND VERNON[31] have reported that reduced PMS or DCIP accelerates the dark recovery of the absorbance changes in chromatophores after continuous illumination. One interpretation of this observation is that the recovery of the optical changes requires reduction of $P870^+$, (oxidized P870), and this is an important point in the argument that the initial absorbance change indicates an oxidation of P870. A demonstration is lacking, however, that the dye is oxidized as P870 recovers, so it remains possible that the dye influences the recovery indirectly.

We were unable to find any evidence that chromatophores do oxidize dyes after a single flash, although continuous illumination causes such an oxidation. At 388 mμ, where oxidized PMS absorbs, the absorbance change induced by a flash in the presence of reduced PMS recovers with an overshoot, which is an A increase at this wavelength (Fig. 9D). However, the same overshoot occurs in the presence of DCIP. Oxidation of DCIP would cause only a negligible A change at this wavelength.

With reduced DCIP present, there is a small overshoot (an A increase) in the recovery at 590 mμ where oxidized DCIP absorbs. But at 588 mμ, only 2 mμ away, there is an overshoot of about the same magnitude in the absence of the dye. At 590 mμ there is a smaller overshoot in the absence of the dye, and at 510 mμ, there is a substantial overshoot. Even if the overshoot at 590 mμ in the presence of DCIP were due entirely to oxidation of the dye, the amount of dye oxidized would be only about 2 % of the amount of P870 which recovers simultaneously.

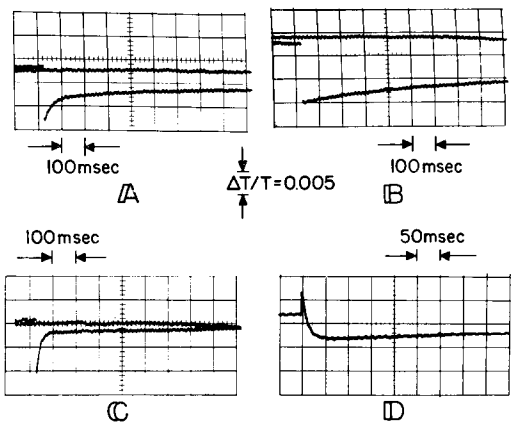

Fig. 9. Effect of oxidized and reduced PMS on the recovery. 295 °K. In this experiment, a cuvette with a 5-mm light path was used. The laser beam struck the clear glass side of the cuvette directly, at a right angle to the measuring light beam. A, 27 μM BChl, 430 mμ, aerobic sample. B, same as A, after the addition of 33 μM PMS. C, same as B, after the addition of 133 μM sodium succinate, bubbling with N_2, and layering with mineral oil. The same effect was obtained without bubbling with N_2 or layering with oil, but the effect was short-lived, unless a larger excess of succinate was added. D, 27 μM BChl; 388 mμ; 17 μM PMS; 133 μM succinate; under N_2 *plus* oil. The overshoot did not depend on the establishment of a steady-state absorbance change by the measuring light: attenuating the beam by a factor of 10 had no effect.

Figs. 9 through 12 present additional information about the optical recovery in the presence of reduced PMS. Fig. 9 shows that the dye increases the portion of the recovery that occurs on the B time scale, but that it does not cause a complete recovery on this time scale. The portion of the absorbance change that remains to recover slowly does not diminish if the PMS concentration is increased, nor does its recovery rate vary markedly with the PMS concentration. Addition of PMS alone, without a reductant, slows and decreases the B scale recovery, again with little effect on the type C recovery (Fig. 9B). Reduced PMS accelerated the recovery at all wavelengths which were examined between 360 mμ and 900 mμ, including 763 mμ. The effect at 763 mμ is of particular interest, because LOACH[25] has shown that treatment of chromatophores with $Na_2S_2O_4$ causes an A increase at this wavelength. If the light-induced A increase at 763 mμ signals the reduction of a chromatophore component, and if PMS accelerates the recovery at other wavelengths by donating electrons to $P870^+$, then reduced PMS should retard the recovery at 763 mμ, not hasten it.

The rapid recovery in the presence of reduced PMS is kinetically first order in the optical change (Fig. 10) and in the concentration of PMS (Fig. 11). From the rate

constants in Fig. 11, one can see that the recovery rate with reduced PMS can be an order of magnitude higher than the rate of cytochrome *c* oxidation after a flash

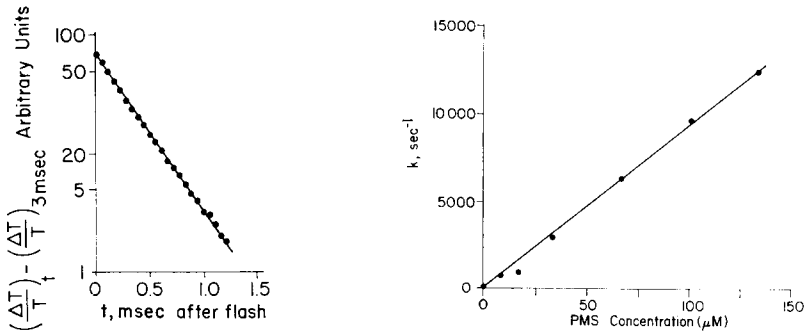

Fig. 10. Kinetics of the rapid recovery in the presence of reduced PMS. 430 mμ, 296 °K, 53 μM BChl, 64 μM PMS, 1.3 mM succinate, under N_2 and oil. $\Delta T/T$ the fractional change in transmittance caused by the flash, was read at 1-mm intervals from a photograph of the oscilloscope screen. After 2 or 3 msec, the recovery entered a slower phase and the trace became essentially flat. $\Delta T/T$ at 3 msec was subtracted from that at earlier times to show the recovery during the rapid step.

Fig. 11. Dependence of the recovery rate on the concentration of PMS. 296 °K, 53 μM BChl, 430 mμ. All samples were under N_2 and oil and contained 1.3 mM succinate. The rate constants were evaluated from plots like that of Fig. 10.

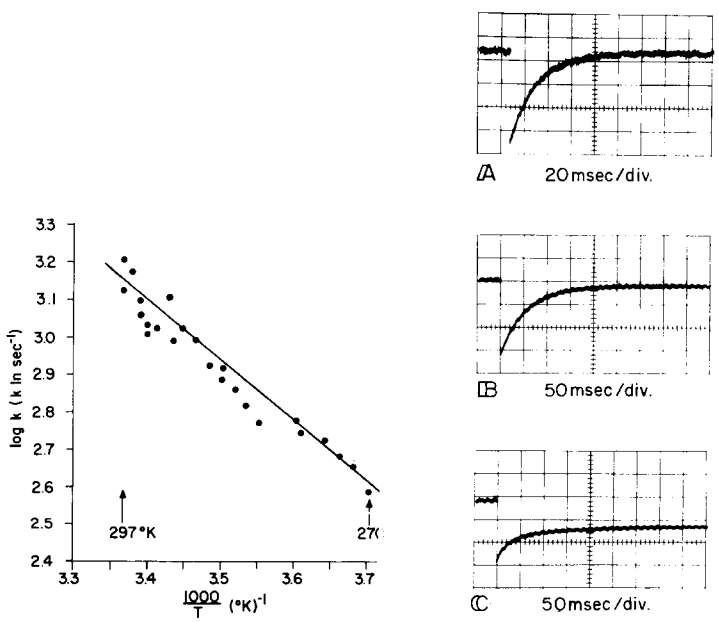

Fig. 12. Temperature dependence of the recovery in the presence of reduced PMS. 430 mμ, 97 μM BChl, 100 μM PMS, 1.5 mM ascorbate, air not removed. The rate constants were evaluated from plots like that of Fig. 10. The log of the rate constant is plotted against the reciprocal of the temperature.

Fig. 13. Flash-induced absorbance changes in frozen suspensions of chromatophores. 126 μM BChl. A, 38 °K; B, 234 °K; C, 252 °K; Note that the sweep rate in A is 2.5 times greater than that in B and C.

in the presence of antimycin. The type A recovery in fresh chromatophores is faster than the recovery given by PMS.

Fig. 12 shows the temperature dependence of the recovery rate with 100 μM PMS and excess ascorbate. The data give a linear Arrhenius plot with $E_a = 10$ kcal per mole. A rapid recovery with PMS and ascorbate occurs even after the chromatophores have been heated aerobically for 10 min at 65°, but the rate is lower by a factor of approx. 2.

The type B recovery is slower if oxidized methyl-red is added. In one experiment, the initial slope of the recovery was 2.34 (ΔA cm$^{-1}\cdot$sec^{-1} at 792 mμ and 295 °K). It decreased to 0.91 upon the addition of 212 μM methyl-red, and increased again to 1.63 upon the addition of 0.6 mM succinate. ASH, ZAUGG AND VERNON[32] have shown that chromatophores can reduce methyl-red in the light if a suitable electron donor is added.

Recovery at low temperatures

AS ARNOLD AND CLAYTON[7] first reported, the light-induced absorbance changes in chromatophores also recover at very low temperatures. Our results differ from those of ARNOLD AND CLAYTON in the kinetic order of the recovery. They found a second order recovery, whereas the low temperature recovery in our experiments was accurately first order (Fig. 13 and Curve A in Fig. 14).

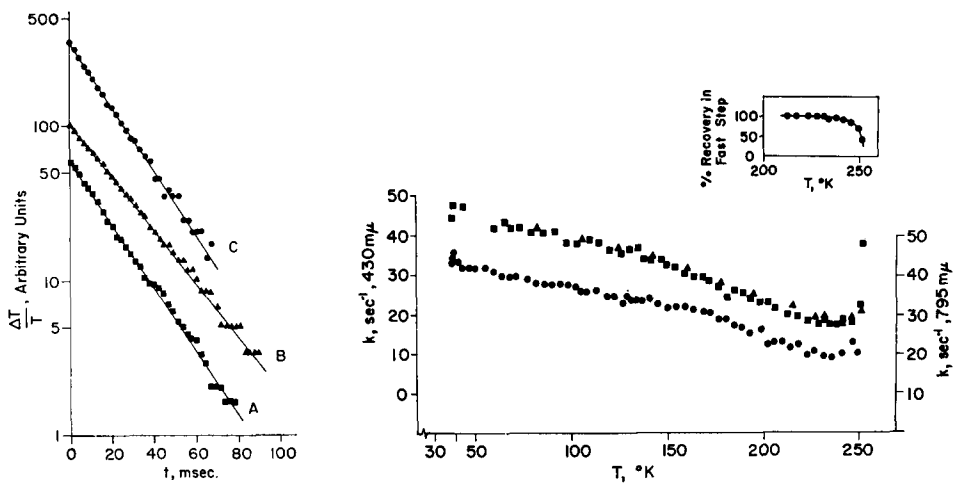

Fig. 14. Kinetics of the recovery at low temperatures. 430 mμ. A, 126 μM BChl; 38 °K; frozen suspension of chromatophores, absorbance change caused by a laser flash. B, 73 μM BChl; 80 °K; frozen suspension of chromatophores, absorbance change caused by 100-msec illumination with red light. A steady state was reached within 50 msec after the shutter opened. C, dried film of chromatophores, laser flash; 80 °K. The points were obtained as in Fig. 10. The curves are displaced arbitrarily along the ordinate.

Fig. 15. Temperature dependence of the recovery rate in frozen suspensions of chromatophores. ■ and ▲, 430 mμ (two different chromatophore preparations); ●, 795 mμ. For clarity, the 430 mμ data are displaced above the 795 mμ data. The rate constants were evaluated from plots like those of Fig. 14. When the recovery was not complete in a single step, only the fast step was analyzed, as described in the legend to Fig. 10. Note that this is not an Arrhenius plot. Both k and T are plotted linearly. Inset: Extent of the recovery in the fast step as a percentage of the total initial absorbance change.

To investigate the reason for this difference, we prepared a dried film of *R. rubrum* chromatophores, similar to the films of *R. spheroides* chromatophores which ARNOLD AND CLAYTON[7] studied. The low temperature recovery in the film was also first order (Curve C in Fig. 14). Our use of a very brief flash did not explain the difference because we obtained a first order recovery after steady-state illumination with red light (Curve B in Fig. 14). (For this experiment, a camera shutter initiated and terminated illumination from a Unitron 9-V lamp with a Corning 2600 filter. The measuring light and electronics of the laser apparatus were used.)

Below 230 °K, the recovery appears to be complete in a single step (Fig. 13). At a given temperature the rate is the same in each of the absorption bands examined (390 mμ, 425 mμ, 430 mμ, 440 mμ, 550 mμ, 570 mμ, 795 mμ, 810 mμ and 895 mμ).

Two observations are relevant to the question of whether a large pool of reductant is responsible for the first order recovery at low temperature. First, laser flashes with saturating light intensity can be repeated many times (at least 30) with the same frozen sample, and the recovery rate shows no tendency to decline or change its kinetic order. The same results were obtained after prolonged steady-state illumination as well. Second, the addition of an oxidant, $K_3Fe(CN)_6$, to the sample before it was frozen diminished the magnitude of the low temperature absorbance change, along with that of the room temperature change, but did not affect the low temperature recovery rate significantly.

For the B type recovery at room temperature and the recovery with reduced PMS, activation energies of 6 and 10 kcal/mole were obtained. Assuming that E_a remains constant at lower temperatures, these reaction rates would be negligible at liquid N_2 temperatures. As expected, the recovery rate at 77 °K was the same whether or not PMS and excess ascorbate were added before the sample was frozen.

Destruction of the room temperature B recovery by heating to 60° had no effect on the recovery rate when the sample was frozen and cooled to 77 °K. Addition of antimycin, HOQNO, or PMA to inhibit the B recovery did not alter the recovery rate at 77 °K.

A remarkable property of the recovery in frozen samples is its temperature dependence. ARNOLD AND CLAYTON[7] and CLAYTON[2] found that the rate in dry films was constant below 150 °K and that above this point the rate decreased with increasing temperature. Figs. 13 and 15 give our results which show the temperature dependence in greater detail. Fig. 15 shows linear plots of the first order recovery rate constant *versus* the absolute temperature. In general, the rate decreases with increasing temperature. At 240 °K, the rate goes through a minimum, and then begins to increase with rising temperature, but the recovery ceases to be complete in a single step. Fig. 15 also indicates the extent of the recovery in the primary step. Fig. 13 illustrates the recovery at representative points.

The change of the rate constant with temperature was completely reversible. It followed the same curve as portions of liquid N_2 were added to the dewar to cool the sample, and as the sample rewarmed toward room temperature. The rate did not depend on whether the sample was cooled abruptly by plunging it in liquid N_2, or gradually with the Joule–Thomson apparatus. The temperature dependence was essentially the same at 430 mμ and 792 mμ, as shown in Fig. 15.

Recovery rates in frozen samples have been measured in 3 different batches of chromatophores, prepared over a period of 5 months and stored at −4° for varying

lengths of time. The rates are surprisingly constant at any given temperature. Fig. 15, which contains data from 3 experiments with 2 different chromatophore preparations, illustrates this point.

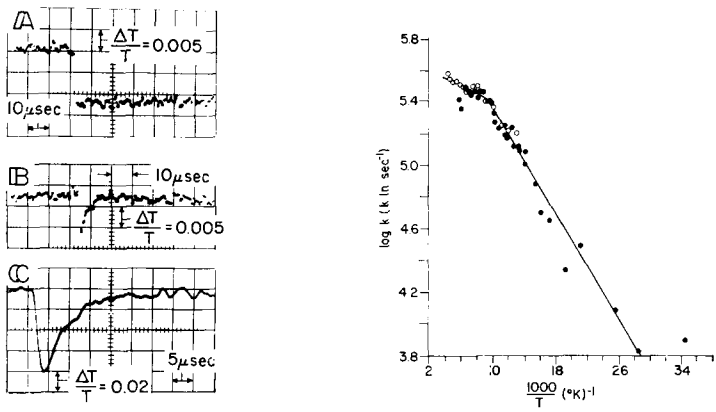

Fig. 16. Effect of $Na_2S_2O_4$ on the absorbance changes. 430 mμ, 81 μM BChl. A, 295 °K, no additions. B, same as A, after addition of several mg/ml solid $Na_2S_2O_4$ and mixing. C, a different sample; 83 °K; solid $Na_2S_2O_4$ added before freezing.

Fig. 17. Temperature dependence of the recovery after addition of solid $Na_2S_2O_4$. The ordinate gives the log of the first order recovery rate constants; the abscissa, the reciprocal of the temperature. Note that the scale on the abscissa is considerably more compact than that in Figs. 5 and 12. This may give a misleading impression of the slope of the curve. The open and closed circles represent experiments with different chromatophore preparations. O—O, 81 μM BChl; ●—●, 119 μM BChl.

Absorbance changes in the presence of dithionite

An important unsolved problem is the identity of the material that first accepts an electron from P870. On the reasoning that one can prevent the oxidation of P870 by reducing the electron acceptor chemically, KUNTZ, LOACH AND CALVIN[5] and LOACH[25] titrated chromatophores with strong reductants such as $Na_2S_2O_4$. This treatment prevented the appearance of the light-induced P870 absorbance changes.

These studies were done with steady-state illumination. Although the authors indicated that acceleration of dark recovery reactions was inadequate to explain the disappearance of the absorbance changes, it seemed possible that strong reductants caused the development of recovery terms too fast for their apparatus to follow. Fig. 16 shows that, in fact, flash-induced absorbance changes do occur in the presence of $Na_2S_2O_4$, but that the dark recovery has a half-time of several μsec. The same phenomenon occurs whether one adds solid $Na_2S_2O_4$ alone (several mg/ml) or indigodisulfonate followed by sufficient solid $Na_2S_2O_4$ just to bleach the dye.

A very rapid recovery in the presence of $Na_2S_2O_4$ also occurs in frozen samples, at least down to 29 °K (Fig. 16C). Apparently, the fast recovery is not due to the formation of a pool of reductant in the frozen chromatophores, or if there is such a pool the recovery does not deplete it permanently because the flashes can be repeated many times and the recovery shows no tendency to diminish in rate. By operating the laser in the normal mode and decreasing the laser beam attenuation, we exposed a frozen sample to approx. 100 flashes of light of intensity similar to that of the single

flash used in Fig. 18. Following this treatment, the sample responded to a single flash with essentially the same recovery rate.

The recovery appears to be kinetically first order, both at room temperature and at low temperatures. Fig. 17 shows the temperature dependence of the recovery rate in frozen samples. Solid $Na_2S_2O_4$ was added just before the samples were frozen. Between 34 °K and 111 °K, the data fit a linear Arrhenius plot with a very small E_a, 380 cal/mole (0.017 eV). Above 111 °K the slope of the experimental curve decreases. However, at this point the half-time is 2.5 μsec, which is approaching the rise time of the preamplifier.

If KBH_4 and indigodisulfonate replaced $Na_2S_2O_4$, we observed an A change and fast recovery at 80 °K but not at room temperature. In this case the absorbance change and recovery may still occur at room temperature but too rapidly for our apparatus to follow.

DISCUSSION

According to the P870 theory of bacterial photosynthesis, the oxidation of P870 should be the most rapid reaction that one can observe following a very brief flash of light, and this should be true even at low light intensities. As cytochrome becomes oxidized, $P870^+$ should become reduced again. The first of these expectations appears to be fulfilled, but there is some doubt about the second. The rapidity of the reaction alone does not prove that $P890^+$ is an intermediate in photosynthesis.

As the recovery kinetics, the dependence on the flash intensity, and the effects on inhibitors are the same at 430 mμ, 792 mμ, and 870 mμ; there is no reason to suppose that more than one pigment undergoes oxidation initially. One should not conclude, however, that the 430 mμ A increase represents an absorption band of oxidized BChl, nor that the absorbance changes at 792 and 870 mμ are due to the same BChl molecule. The oxidation of a single BChl molecule could affect the absorption spectra of neighboring molecules indirectly.

The effect of antimycin on the spectral changes seems to show that cytochromes *b* and *c* participate in the return of electrons to P870. Studies with continuous illumination have had difficulty proving this point for *R. rubrum* chromatophores, although SMITH AND BALTSCHEFFSKY[6] have obtained evidence for the involvement of cytochrome *c*. Part of the difficulty in observing cytochrome *c* oxidation in *R. rubrum* chromatophores appears to arise because this process, rather than the dark reduction, is much slower than it is in whole cells. The provision of a long dark period preceding the flash, and the addition of reductants such as succinate, glutathione, or reduced PMS did not make the cytochrome oxidation more conspicuous. Of course, the superposition of the A changes due to cytochrome and those due to the 430 mμ pigment also impedes observation of the cytochrome oxidation unless one can separate them temporally.

The available evidence is insufficient to settle whether or not the recovery of P870 after a flash actually involves reduction of $P870^+$ by cytochrome *c*. Fig. 7 indicates that part of the P870 does recover at approximately the same rate as cytochrome *c* is oxidized, but the absorbance changes are too small to indicate whether the kinetics are identical. The amount of P870 that recovers appears to be twice as great as the amount of cytochrome which is oxidized, but there are uncertainties

in the absorbance coefficients as well as the measurements. We are investigating this problem in *Chromatium* and other systems in which cytochrome oxidation is more pronounced.

There are two plausible interpretations of the A (20 μsec) recovery. First, it could be due to a portion of the cytochrome *c* which retains an undamaged relationship to P870. This might account for its sensitivity to heating and aging. Alternatively, the recovery could be due to a back reaction by a different route, similar to the recovery which occurs at low temperatures. A back reaction might also be sensitive to aging or heating although the low temperature recovery proceeds in aged or heated chromatophores which have lost the A recovery.

The B recovery consists of multiple kinetic terms, perhaps in part because the chromatophore preparation is heterogeneous, and in part because it involves electron transport over several steps. Chemical oxidation of some of the carriers, as by the addition of oxidized methyl-red or PMS, hinders the recovery. With steady-state illumination, KUNTZ, LOACH AND CALVIN[5] found that shifts of the redox potential of the milieu changed the extent and rate of the recovery on this time scale. The more reducing the potential, the faster and more complete was the recovery.

The nature of the very slow (C) recovery at room temperature remains an enigma. The slow step is insensitive to inhibitors and to heating; it can be diminished in importance, but not eliminated, by the addition of reduced PMS. Possibly, an understanding lies in heterogeneity of the sonicated preparation. Disruption of the bacterial cell may separate part of the P870 from other molecules which are essential for the recovery reactions. We have not attempted any fractionation, but FLEISCHMANN[33] has found differences between heavier and lighter fractions of *Rps. spheroides* chromatophores.

Reduced PMS appears to react, not with cytochrome *c*, but more directly with P870 because the recovery rate can be greater than the rate of cytochrome *c* oxidation as measured in the presence of antimycin. This reasoning could be in error if there is a special fraction of cytochrome which is responsible for the A recovery and if PMS reacts with this cytochrome. We cannot dismiss this possibility at present although PMS is still active in aged or heated preparations which appear to have lost the A recovery.

There are several possible explanations why we could not detect oxidation of PMS or DCIP following a flash. The reaction of PMS with $P870^+$ might involve several steps so that oxidation of the dye is too slow to measure with our apparatus. Possibly, the dye is reduced again as rapidly as it is oxidized. Or the dye might be oxidized to a semiquinone which absorbs at an unexpected wavelength.

Although these suggestions have some plausibility, our failure to observe oxidation of the dyes means that the effects of oxidants and reductants on the recovery rates may be indirect, and this weakens the evidence that P870 is oxidized in the primary photochemical reaction. That chemical oxidants and light produce similar absorbance changes is not completely conclusive because, as KAMEN[34] has emphasized, reduction could have a similar effect on the BChl absorption spectrum. The formation of a BChl triplet or some other excited species might cause similar absorbance changes, but the very long lifetime of the absorbance changes, their insensitivity to O_2, and the fact that heating or adding inhibitors makes the recovery even slower all suggest that a true chemical reaction has occurred.

Assuming that the initial absorbance changes do reflect an oxidation of a specialized chlorophyll, the recovery at low temperature evidently involves a back reaction by a route which does not involve the cytochromes. It is insensitive to the inhibitors which block cyclic electron transport. It is unaffected if the room temperature A and B recoveries are damaged by heating. Further, the room temperature recoveries (including that provided by PMS, but not necessarily including the A recovery, for which we do not have accurate measurements) have large enough activation energies so that their rates would be negligible at 77 °K. Of course, it is impossible to make these arguments logically complete because a site which is sensitive to inhibition in solution may be insensitive in a frozen sample. Activation energies are not necessarily constant over an extended temperature range which includes phase transitions. Nevertheless, interpretation of the low temperature recovery as a back reaction encompasses the intriguing observation of MAYNE AND CLAYTON[8] that the low temperature *A* changes do not occur if one illuminates the sample while cooling it. At intermediate temperatures the electron apparently escapes from the region of P870 over an energy barrier. From there the electron can return to $P870^+$ only by completing a cyclic path, involving steps which are vulnerable to inhibitors and which require additional activation energy. At low temperatures lack of the necessary activation energy prevents the electron from entering this path.

If the initial photochemical reaction generates a reductant (X^-) and an oxidant ($P870^+$) in equal concentrations, one might expect the low temperature recovery to be first order in each of the reactants and thus to appear second order. In dried films of *Rps. spheroides* chromatophores, ARNOLD AND CLAYTON[7] did observe a second order recovery at low temperatures. There are several ways to account for the first order recovery in our system. One is to assume that X^- and $P870^+$ are both immobile so that reactions can occur only between fixed pairs. Another possibility is that X^- is present in great excess over $P870^+$ or is in equilibrium with a reductant present in excess, but this seems improbable because the recovery can be repeated many times and because the addition of $K_3Fe(CN)_6$ does not affect the low temperature recovery rate. Finally, first order kinetics could result if there are several steps in the recovery. For example, the rate-determining step could be a reaction between X^- and an intermediate carrier, which then passes an electron rapidly to $P870^+$.

One must also explain why the low temperature recovery rate increases as the temperature falls and why the recovery becomes very much faster upon the addition of $Na_2S_2O_4$. One way to account for the temperature dependence is to assume that the electron of X^- equilibrates between X and another material, R. R could be an unproductive trap, as ARNOLD AND CLAYTON[7] have suggested. If X^-, but not R^-, can react with $P870^+$ or with an intermediate carrier, the equilibrium will reduce the effective concentration of X^- and slow the recovery. If the reaction $X^- + R \rightarrow X + R^-$ has a higher activation energy than the reverse reaction does, the equilibrium will shift toward X as the temperature falls, increasing the recovery rate.

It might appear that this hypothesis can explain the effect of $Na_2S_2O_4$ as well. If $Na_2S_2O_4$ reduces R chemically, so that the equilibrium cannot lower the concentration of X^-, one would observe the true rate of the back reaction between X^- and $P870^+$. Then the observed rate would depend on temperature according to the Arrhenius equation. But there is a paradox in this reasoning. If the equilibrium shifts toward X^- as the temperature falls, it must favor X^- at all temperatures. But if

this is so, then $Na_2S_2O_4$ should reduce X along with R, and the light-induced absorbance changes should disappear. One could escape this difficulty by assuming that R drains off electrons, not from X^-, but at some intermediate step in the recovery.

There are other possibilities for explaining the temperature dependence and the effect of $Na_2S_2O_4$. Strong reductants might generate a material which can serve as an effective intermediate carrier for the returning electron (or hole). Alternatively, they might cause structural changes that bring such a carrier into a new position, where it can act most effectively. If a slow step were limited by the geometrical arrangement of the reactants, and if the geometry changes with temperature, this might explain the temperature dependence of the reaction rate. It might also explain why the back reaction becomes insignificant at room temperature. If the recovery reaction involves electron tunneling through a barrier[35], the rate would be sensitive to the matching of energy levels on either side of the barrier. The electronic energy levels of P870 and B890 obviously change as the temperature falls because the absorption spectra change.

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