BBA 46056

OXIDATION–REDUCTION POTENTIAL DEPENDENCE OF THE INTERACTION OF CYTOCHROMES, BACTERIOCHLOROPHYLL AND CAROTENOIDS AT 77°K IN CHROMATOPHORES OF *CHROMATIUM* D AND *RHODOPSEUDOMONAS GELATINOSA*

P. LESLIE DUTTON

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)

(Received July 13th, 1970)

SUMMARY

1. A technique for assay of light-induced reactions at 77°K as a function of oxidation–reduction potential has been developed.

2. The light-induced reactions at 77°K in *Rhodopseudomonas gelatinosa* and *Chromatium* D have been studied as a function of oxidation–reduction potential imposed on the chromatophores before freezing.

3. In *Chromatium* D, cytochrome C553 (E_m +10 mV) is oxidized by the bacteriochlorophyll reaction center complex (E_m +470 mV) which undergoes light-induced bleaching at 615 nm, 430 nm and 400 nm; these spectral components are probably part of the reaction center P890 (P883). The evidence suggests that either of two cytochrome C553 hemes are capable of transferring electrons to bacteriochlorophyll$^+$ at 77°K. The primary electron acceptor associated with the reaction center bacteriochlorophyll has E_m —135 mV.

4. In *R. gelatinosa*, cytochromes C422 (E_m +280 mV) and C419 (E_m +128 mV) are both apparently oxidized by a reaction center bacteriochlorophyll, E_m +400 mV; the molar ratio of these three components reacting at 77°K is 0.55:1:1, respectively. The primary electron acceptor of this system has E_m —140 mV. However, the fact that some cytochrome oxidation was detectable at —350 mV indicates another and lower potential acceptor.

5. Evidence in the cited species indicates that a single reaction center oxidizes both the high and low potential cytochromes.

6. As the ambient oxidation–reduction potential is decreased, the control of cytochrome C422 oxidation in *R. gelatinosa* appears directly associated with the oxidation–reduction curve of the low potential cytochrome C419.

7. Carotenoid absorbance changes observed in *R. gelatinosa* at 77°K appear to respond to the light-induced oxidation–reduction reactions of reaction center bacteriochlorophyll, the primary electron acceptor, and to the cytochromes; the carotenoid changes may result from electrostatic field alterations.

Abbreviations: E'_0, the midpoint oxidation–reduction potential of a couple at pH 7. E_m, the midpoint under other specified conditions[22].

INTRODUCTION

The effects of chemically imposed oxidation–reduction potentials on the light-induced reactions of the electron transport components in photosynthesis have been studied by several workers[1–7]. CUSANOVICH *et al.*[3], in accordance with the finding of PARSON[8], proposed that the reaction center bacteriochlorophyll component, $P890^+$, E_m +490 mV[3] (recently more precisely termed $P883^+$ (see ref. 27)), was directly responsible for the oxidation of the high oxidation–reduction potential cytochrome C555 (E_{m7} +330 mV (see ref. 3)); they further indicated that the low potential cytochrome C553 (E_{m7} +10 mV (see ref. 9)) was oxidized in the light by a second reaction center bacteriochlorophyll. The postulation that more than one reaction center operates in bacterial photosynthesis is consistent with the experiments of MORITA[10] and SYBESMA AND FOWLER[11–13], with *Chromatium* D and *Rhodospirillum rubrum*, respectively. However, recent laser studies[14,15] have suggested that P883 oxidizes both cytochromes. Cytochrome C553 appears to be in a non-cyclic electron pathway and cytochrome C555 in a cyclic electron transfer system of *Chromatium* D[16].

This paper describes the effect of oxidation–reduction potentials on light-induced electron flow in chromatophores of *Chromatium* D and *R. gelatinosa* at liquid nitrogen temperatures. At these low temperatures, electron transfer appears to be limited to components chemically integrated with the reaction center bacteriochlorophyll; that is, its primary electron acceptor and those cytochromes which are electron donors to the light generated bacteriochlorophyll$^+$. In *Chromatium* D chromatophores, the cytochrome which is photooxidized at 77°K appears to be cytochrome C553 since both cytochrome C553 and the low temperature cytochrome are readily oxidized by oxygen[17]. In *R. gelatinosa* chromatophores at 77°K, two cytochromes undergo apparently independent photooxidation[18] and appear to behave analogously to the cytochromes C553 and C555 in *Chromatium* D at room temperature[3,13,14]. Study of light-induced reactions at 77°K as a function of oxidation–reduction potential in chromatophores of these two photosynthetic bacteria offered the opportunity with *Chromatium* D to study uniquely the relationship of C553 with its reaction center bacteriochlorophyll. With *R. gelatinosa* it provided the means to determine: (a) the factors controlling the photooxidation of the two cytochromes; (b) whether the cytochromes are associated with the same or separate reaction center bacteriochlorophyll; and (c) what govern the light-induced responses of carotenoids at 77°K.

Preliminary reports of this work have been presented[19,20].

MATERIALS AND METHODS

R. gelatinosa (strain I) were grown anaerobically in the light as previously described[18]. *Chromatium* D (American Type Culture Collection) was grown autotrophically in medium described by MORITA *et al.*[21]. They were harvested toward the end of the exponential growth phase; *R. gelatinosa* after 35–40 h, and *Chromatium* D after 80-90 h. The cells were washed once with 0.1 M Tris–HCl buffer, pH 7.4, and resuspended in 15 ml of the same buffer containing 6–7 g wet wt. *R. gelatinosa* or 3–4 g wet wt. *Chromatium* D. The cells were disrupted by ultrasonic oscillation (Branson Sonifier, Type S75, output 3.5 d.c. A; 2 min at 0–4°) and the cell debris removed by centrifugation (2°; 3 times at 30000 × *g* for 20 min). Further centrifugation (2°; 105000 × *g*

for 60 min for *Chromatium* D, or 120 min for *R. gelatinosa*) sedimented the chromatophores which were washed twice with 0.1 M Tris–HCl buffer, pH 7.4, suspended in a minimal volume of this buffer and stored at 0–2° under a nitrogen atmosphere.

Light-induced absorbance changes were followed in a Johnson Foundation dual wavelength spectrophotometer equipped with a clear-sided Dewar flask for work at liquid nitrogen temperatures. The sample cuvette was illuminated normal to the measuring beams with near infrared light from a tungsten source filtered through a Wratten 88A filter. A Corning blue-green filter (9788) protected the photomultiplier (E.M.I. 9524B) from the near infrared cross illumination.

The apparatus used for the oxidation-reduction potential titrations of light-induced reactions are shown in Figs. 1 and 2.

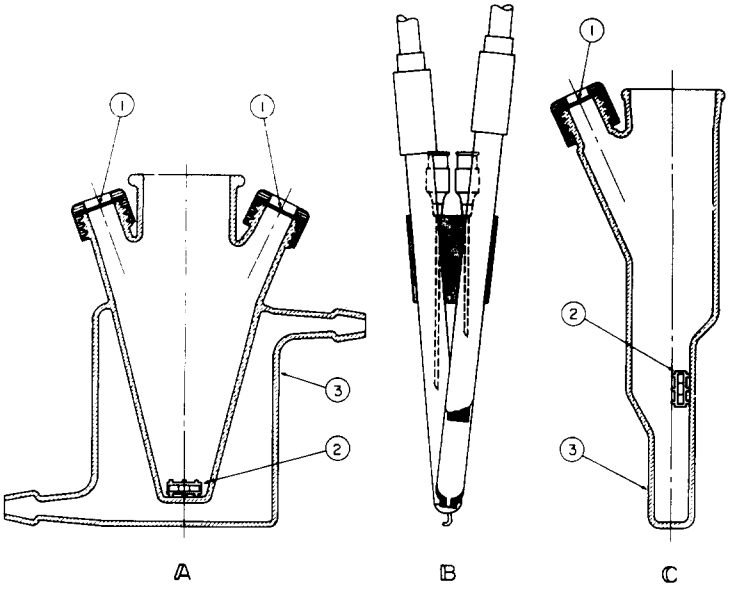

Fig. 1. Apparatus for oxidation–reduction potential measurements. A. Reaction vessel: (1) septum; (2) magnetic stirrer; (3) water jacket. B. Stopper for vessels A and C containing electrode assembly and gas inlet and outlet hypodermic needles. C. Reaction cuvette for simultaneous dual wavelength spectrophotometric assay: (1) septum; (2) magnetic stirrer; (3) 1-cm optical path. For details, see text.

The reaction vessel (Fig. 1A) was fitted with a water jacket at 295°K. The shape of the interior was conical, providing a maximum depth-to-volume ratio for accomodation of the electrodes in small volumes; the minimum volume which could be used was 2 ml and the capacity volume was 50 ml. The contents were stirred from the bottom with a magnetic stirrer. The vessel was fitted with side-arms sealed with septums (Hamilton Co.; Type 18288) through which additions could be made and samples withdrawn. The vessel was sealed with a silicone rubber stopper (A. H. Thomas Co., Philadelphia), which also accomodated the electrodes, and a gas inlet and outlet. The oxidation–reduction potential was measured using a platinum electrode (Radiometer, Copenhagen; Type P101) and a standard calomel electrode (Radiometer, Copenhagen; Type K401). The contents were maintained under an argon atmosphere which contained less than 1 ppm O_2 (Matheson Co., Ultra High Purity grade), introduced

through the stopper *via* hypodermic syringe needles fitted with Kel-F luer hubs (Hamilton Co., Gauge 24). Teflon tubing (Hamilton Co.) having an internal diameter of 1.42 mm and a total length of 2 ft, was used to convey the gas to the vessel *via* a bubbler containing water which saturated the gas with water vapor. The gas outlet passed through an 8 cm head of water, thus creating a small positive pressure in the system. All connections for the gas train were Kel-F luer fittings (Hamilton Co.). Small additions were made with 10-μl syringes (Hamilton Co.). Samples of chromatophores were withdrawn in a 1-ml gas tight syringe with a modification that enabled it to be flushed with inert gas before use (Hamilton Co., Type GF1002). Samples were immediately transferred to the cuvette shown in Fig. 2 for study at liquid nitrogen temperatures.

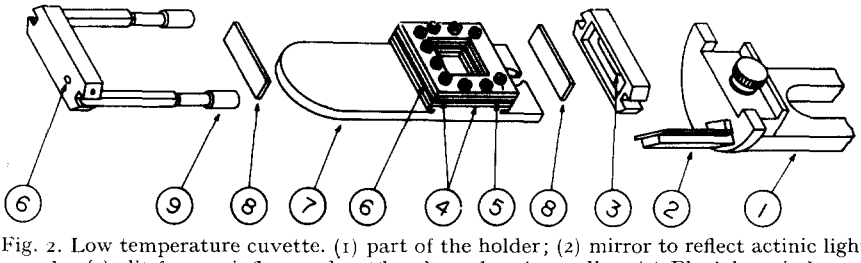

Fig. 2. Low temperature cuvette. (1) part of the holder; (2) mirror to reflect actinic light onto the sample; (3) slit for gas inflow and outflow hypodermic needles; (4) Plexiglas windows; (5) 1-mm spacer; (6) orifice for introduction of chromatophore sample *via* hypodermic syringe; (7) tongue to prolong the contact of the cuvette with liquid nitrogen; (8) septum; (9) screw to clamp septum and end pieces firmly to the cuvette. For details, see text.

The low temperature cuvette (Fig. 2) was gas tight. The light path was 1 mm, and its capacity volume was 0.3 ml. The top of the cuvette was open and there was a small orifice in the bottom; both openings were covered with rubber septums (1/16 Teflon rubber sheeting) clamped firmly in position. The cuvette was thoroughly flushed with nitrogen (Matheson Co., prepurified grade) through the top septum using syringe needles (Hamilton Co., Gauge 27). The sample of chromatophore suspension was introduced into the cuvette through the bottom septum so the cuvette was filled in an upward direction to avoid the formation of bubbles. The cuvette was then screwed onto the holder and plunged into liquid nitrogen contained in the Dewar in the spectrophotometer.

The main reaction vessel was covered with a light-tight black jacket and all transfer procedures were done in near darkness.

Fig. 1C shows the cuvette which was used at room temperature for continuous assay of the absorption changes with simultaneous oxidation-reduction potential measurement. The cuvette could also accomodate the stopper assembly of Fig. 1B. The contents (5–6 ml) were stirred by a magnet that was maintained on the inside wall of the vessel and rotated by a magnetic rotor placed close to the outside of the vessel.

The equipment was tested in several ways. Readings of the electrodes and pH/mV meter were within 2 mV of the known[22] oxidation–reduction potential of saturated solutions of recrystallized quinhydrone (Fisher Company) at pH 6.0 and 7.0. In the presence of neutral red (100 μM), the drift of the oxidation–reduction potential readings of chromatophore as low as —340 mV were of the order 3–4 mV/min; at —125 mV in the presence of potassium indigotetrasulphonate (100 μM) the drift was extremely low. Using the gas tight syringe, repeated withdrawl and return of 1 ml chromato-

phore suspension from a total volume of 3 ml of chromatophores poised at these negative oxidation–reduction potentials under experimental conditions did not significantly change the oxidation–reduction potential reading. Using benzyl viologen and anthroquinone 2-sulphonate as mediators, the E'_0 of horse radish peroxidase (Worthington Biochemicals Co.) was —260 mV (one electron transfer) which was close to the value obtained by HARBURY[23] (experiment performed in collaboration with Drs. M. Waterman and T. Yonetani).

Over the oxidation–reduction potential range of +500 mV to —350 mV, a variety of oxidation–reduction chemicals were employed to mediate between the platinum electrode and the electron carriers contained in the chromatophore particles. The mediators used included combinations of those which were proven successful in the experiments of CUSANOVICH *et al.*[3] and CRAMER AND BUTLER[4].

The combination of mediators were: (A) potassium ferri-ferrocyanide (E'_0 +430 mV), 100 μM ferricyanide added at the start; (B) dichlorophenolindophenol (E'_0 +217 mV), 40 μM; (C) *N,N,N'N'*-tetramethylphenylenediamine (E'_0 +260 mV), 40 μM; (D) ethylenediaminetetraacetic acid, 10 mM, and ferric chloride, 300 μM (E'_0 +117 mV); (E) potassium oxalate, 10 mM, and ferric chloride, 300 μM (E'_0 +2 mV), potassium indigotetrasulphonate (E'_0 —46 mV), 100 μM, potassium indigosulphonate (E'_0 —125 mV), 100 μM; (F) sodium anthraquinone 2-sulphonate (E'_0 —225 mV), 100 μM, 2-hydroxy-1,4-naphthaquinone (E'_0 —145 mV), 100 μM; (G) as (F) with neutral red (E'_0 —325 mV), 100 μM; (H) as (G) with 5-hydroxy-1,4-naphthaquinone (E'_0 +30 mV), 70 μM, and 1,4-naphthaquinone (E'_0 +60 mV), 70 μM.

The oxidation–reduction potentials of the chromatophore suspensions were made more positive with potassium ferricyanide (100 mM), and more negative by either allowing the endogenous reducing system of the chromatophores to act, or by the addition of a freshly prepared solution of sodium dithionite in 0.1 M Tris–HCl buffer, pH 7.8.

The reaction vessel and 0.1 M Tris–HCl buffer, pH 7.4, containing the appropriate mediators were thoroughly flushed with argon to remove oxygen before the addition of the chromatophores. A different experiment using the same concentration of chromatophores was conducted for each combination of mediators. In any one experiment, samples for analysis were taken at 77°K over the mediated potential range; first in a reductive phase and then in an oxidative phase of the titration to establish that the system was reversible and near equilibrium. The time allowed for equilibrium to be attained after adjustment of the oxidation–reduction potential was at least 6 min. In general, the mediators were effective; one exception was the ferri–ferrooxalate with which marked deviations were observed above +40 mV. In Figs. 3 and 4 the points (●) were taken during an oxidative sequence. A subsequent examination of a reductive sequence revealed a deviation in the other direction.

Bacteriochlorophyll concentration was estimated as described previously[14].

The intensity of the near infrared actinic light was sufficient to saturate the reactions measured at 77°K.

RESULTS

Chromatium D

The light-induced difference spectrum (370–630 nm) of anaerobic crude chromatophore preparations of *Chromatium* D at liquid nitrogen temperatures is due to the

photooxidation of a cytochrome which produces a λ_{max} 408 nm and a λ_{min} 421 nm in the Soret, and a λ_{min} 550 nm in the α-band region[24]. The photooxidation is completely non-reversible (*cf.* ref. 25). Aeration of the chromatophores abolishes the cytochrome reaction which is replaced by reversible light-induced absorbance changes throughout the visible region which are attributable to bacteriochlorophyll photooxidation and dark reduction[24]. These changes displayed absorption λ_{min} at 385 nm and 400 nm (P400), a broad λ_{max} centered at about 430 nm (P435), and a λ_{min} at 615 nm with a shoulder at 600 nm (P600).

The experimental procedure for the assay of the light-induced reactions as a function of oxidation-reduction potential was to first measure the extent of absorbance decrease due to cytochrome oxidation at 421 nm since the reaction was non-reversible. Any reversible absorbance changes due to bacteriochlorophyll oxidation at 430 nm were then measured at 421 nm (at this wavelength the absorption increase measured was 70 % of that occurring maximally at 430 nm), 615 nm, 600 nm, and 400 nm. The operation took several minutes but over this time period it was noted, using a thermo-couple, that the temperature remained in the region of 77 °K.

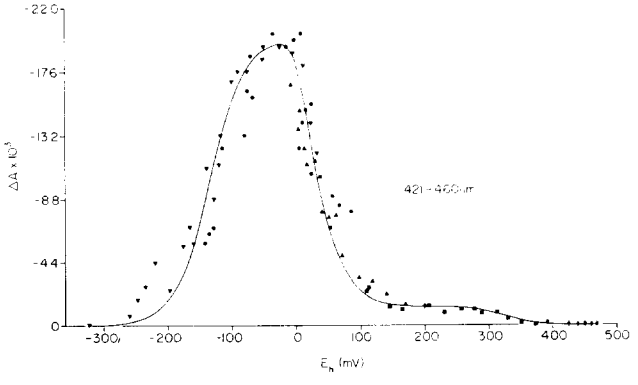

Fig. 3. Photooxidation of cytochromes in *Chromatium* D chromatophores at 77 °K as a function of oxidation–reduction potential. The symbols represent different mediator combinations: ◆, A; ■, C; ▲, D; ●, E; ▼, H. Bacteriochlorophyll concentration, 270 μM. Light path 1 mm.

Fig. 3 shows the oxidation–reduction potential profile of cytochrome photooxidation at 77 °K in *Chromatium* D chromatophores. Between potentials of +380 mV and +260 mV, a cytochrome with E_m +325 mV becomes chemically reduced; the small extent of photooxidation observed at 77 °K is non-reversible. The similarity of the E_m of this cytochrome to that of cytochrome C555 (see ref. 3) is an indication of partial cytochrome C555 photooxidation at 77 °K. The amplitude of the change, however, is less than 5 % of that which is capable of undergoing photooxidation at room temperature at similar potentials and equivalent bacteriochlorophyll concentrations.

At oxidation–reduction potentials lower than +150 mV more extensive changes become evident. Initially the course of oxidation–reduction of this cytochrome closely follows that expected for a theoretical one-electron curve with E_m +10 mV. Cytochrome C553, isolated from *Chromatium* D, has E_m +10 mV (9). However at oxidation–reduction potentials below 0 mV, the amplitude of the photooxidation of this cytochrome ceases to increase with decreasing potential. This would be expected if there were more than one cytochrome C553 heme complexed with, and able to trans-

fer electrons to, the reaction center bacteriochlorophyll molecule. This is consistent with the presence of several (4–7) cytochrome C553 hemes per reaction center bacteriochlorophyll[14,27] in each *Chromatium* D photosynthetic unit. The experimental points fit closest to a theoretical curve derived assuming that only two of the cytochrome C553 hemes are capable of transferring electrons to the reaction center bacteriochlorophyll$^+$ molecule at 77°K; it may be envisaged that the two hemes perhaps belong to the one cytochrome C553 molecule which is complexed directly with the reaction center bacteriochlorophyll. The hemes of the other cytochrome C553 molecules in the photosynthetic unit may be unfavorably placed for electron transfer to the bacteriochlorophyll$^+$ at 77°K. The theoretical curve is drawn assuming that there is no energy transfer between photosynthetic units[14,31], that there is no electron transfer at 77°K between the hemes of different cytochrome C553 molecules in the photosynthetic unit, and that the E_m of the cytochrome hemes is +10 mV. It is appreciated, however, that if the E_m is —10 mV or —25 mV, then the experimental points can accomodate a theoretical curve almost as well, assuming that 4 or 6 hemes, respectively, could transfer electrons to the bacteriochlorophyll$^+$ molecule either directly or *via* other cytochrome C553 molecules.

From —50 mV to —300 mV the extent of cytochrome oxidation diminishes, the course of attenuation following a theoretical one-electron curve with E_m —135 mV. This may be attributed to chemical reduction of the primary electron acceptor of bacteriochlorophyll, which prevents bacteriochlorophyll$^+$ formation in the light.

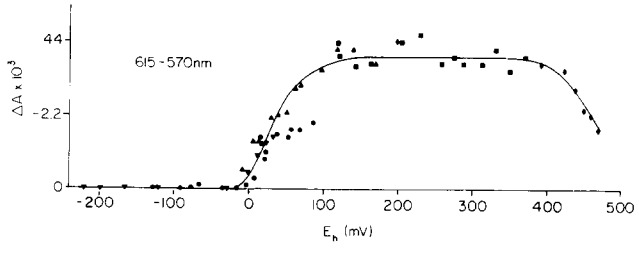

Fig. 4. Photooxidation of bacteriochlorophyll at 615 nm (P600) in *Chromatium* D chromatophores at 77°K as a function of oxidation–reduction potential. Details are given in Fig. 3.

The role of bacteriochlorophyll in light-induced electron flow at 77°K is shown in Fig. 4. This describes the reversible reaction of bacteriochlorophyll photooxidation and dark reduction monitored at 615 nm (P600). This is the second transition band of bacteriochlorophyll at 77°K. No non-reversible absorption changes at 615 nm were detected when examined at widely different oxidation–reduction potentials. (It is important to note that when the light generated bacteriochlorophyll$^+$ measured does accept an electron from cytochrome C553, it will have done so in 2–3 msec (see ref. 30), which is more rapid than the response time of the dual wavelength spectrophotometer. The light-induced absorption change due to bacteriochlorophyll photooxidation, therefore, is a measure of the bacteriochlorophyll$^+$ in photosynthetic units which contain no reduced cytochrome or reduced cytochrome incapable of undergoing photooxidation at 77°K.) From +380 mV down to +100 mV the extent of the bacteriochlorophyll photooxidation remains fairly constant. The course of attenuation between +100 mV and 0 mV is proportional to the extent of cytochrome C553 photooxidation. The line drawn through the points is derived from the same theoretical

oxidation–reduction curve of cytochrome C553 used in Fig. 3, thus demonstrating that cytochrome C553 is oxidized by P600. The midpoint oxidation–reduction potential of the P600 is +470 mV and fits the theoretical one-electron curve drawn through the points.

The reactions monitored at 600 nm, 421 nm (P435) and 400 nm as a function of potential are within experimental error identical to those observed at 615 nm, suggesting that these absorption bands are closely associated with, or are spectral constituants of the same bacteriochlorophyll molecule. CUSANOVICH *et al.*[3] obtained an E_m value of +490 mV for reaction center bacteriochlorophyll determined at 808 nm (P800) and 883 nm (P883) in *Chromatium* D. The similarity of this value to those of P600, P435 and P400 would extend the association to include these more extensively studied component peaks of the oxidized-*minus*-reduced spectrum of reaction center bacteriochlorophyll. These observations indicate, therefore, that the P800:P883 complex, known to be the oxidant of cytochrome C555[8], also effects the oxidation of cytochrome C553 and suggests that one reaction center bacteriochlorophyll functions in *Chromatium* D for the oxidation of both cytochrome C555 and C553.

R. gelatinosa

Similar experiments were performed using chromatophores of *R. gelatinosa*. This organism is of interest because it contains two cytochromes capable of undergoing photooxidation at 77°K[18]. Fig. 5 shows the light-*minus*-dark spectra of the two cytochromes. Cytochrome C419 photooxidation occurs in crude chromatophore preparations kept under anaerobic conditions before freezing to 77°K. This cytochrome, like cytochrome C553 in *Chromatium*, is non-reversibly photooxidized. In the presence of oxygen *R. gelatinosa* extracts display the light-induced oxidation of a different cytochrome: cytochrome C422. Unlike cytochrome C419, cytochrome C422 appears to be reversibly reduced in the dark at 77°K after photooxidation. Under aerobic conditions there is a light-induced absorbance increase centered at 435 nm and a decrease centered at 610 nm, characteristic of bacteriochlorophyll. The organism also displays carotenoid absorbance changes which are non-reversible under anaerobic conditions and reversible in the presence of oxygen[18,24].

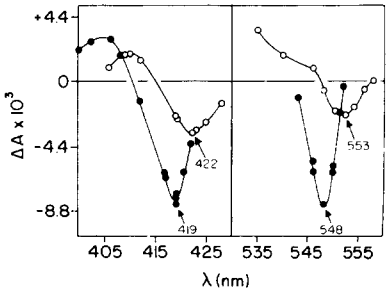

Fig. 5. Light-induced difference spectra at 77°K of cytochrome C419 and cytochrome C422 in unwashed chromatophores of *R. gelatinosa*. ●, anaerobic conditions before freezing, non-reversible cytochrome C419 photooxidation; ○, *plus* subsaturating oxygen, reversible cytochrome C422 oxidation. Bacteriochlorophyll concentration, 350 μM. Light path 1 mm.

(a) Cytochrome and bacteriochlorophyll reactions

Fig. 6 shows the light-induced absorbance changes in the Soret spectral region as a function of oxidation–reduction potential. The course of oxidation–reduction of

cytochrome C422 is close to a theoretical one-electron curve with E_m +280 mV. As the oxidation–reduction potential was lowered (+200 mV to +50 mV), cytochrome C422 photooxidation becomes attenuated by about 85–90 % fitting a one-electron curve with an E_m +135 mV. The course of attenuation coincides with the increase of

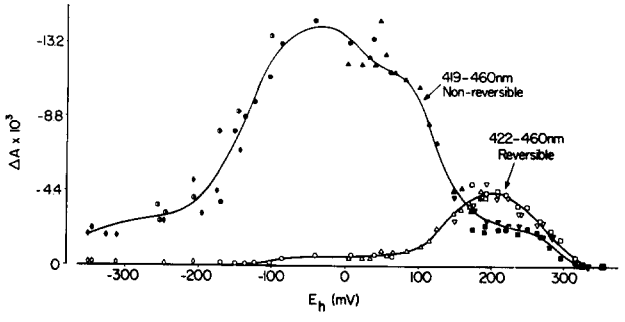

Fig. 6. Photooxidation of cytochromes in *R. gelatinosa* at 77 °K as a function of oxidation–reduction potential. The open symbols are the reversible reactions, and the closed or semi-closed symbols are the non-reversible reactions for the different mediator combinations: ■, B; ▼, C; ▲, D; ●, E; ◑, F; ◆, G. Bacteriochlorophyll concentration, 185 μM. Light path 1 mm.

non-reversible cytochrome C419 photooxidation (see Fig. 9), which becomes chemically reduced in this range with an E_m +128 mV, the course of its oxidation–reduction also fitting a theoretical one-electron curve. It is clear in Fig. 6 that at potentials too high for C419 photooxidation to be significantly observed, there is a second, non-reversibly photooxidized cytochrome. The E_m of the oxidation–reduction curve of the second non-reversible reaction is similar to that of the reversible C422 reaction, implying that a significant proportion of cytochrome C422 was non-reversibly oxidized at 77 °K. This has been calculated to be 45 % of the total C422 photooxidizable at 77 °K. The remainder is repeatably photooxidizable, and many light on/off transitions do not diminish the extent of this reaction.

The kinetics of the non-reversible cytochrome oxidation at 419 nm change below 0 mV. In addition to the immediate response on the dual wavelength spectrophotometer recorder due to cytochrome C419 oxidation, there is an additional slow reaction with a half-time of about 15 sec. The light-induced difference spectrum of this component is not very different from that of cytochrome C419, since light-induced spectra between 400 nm and 560 nm, including the slow component, are similar to that for the fast C419 reaction alone.

From —150 mV to —220 mV the extent of cytochrome photooxidation is attenuated. The course of attenuation fits a one-electron curve with an E_m of about —140 mV. As with *Chromatium* D, the attenuation can be interpreted as the result of the chemical reduction of the observed primary electron acceptor of bacteriochlorophyll. However, the slow component is still evident at —350 mV indicating the existence of another and much lower oxidation–reduction potential, primary electron acceptor from bacteriochlorophyll; its E_m would be lower than —350 mV. The amplitude of the slow oxidation reaction at these potentials is approximately the same as the enhancement seen around 0 mV, which would suggest that the faster cytochrome C419 oxidation is completely attenuated with E_m —140 mV.

The reversible absorbance changes at 610 nm due to bacteriochlorophyll photo-

oxidation are described in Fig. 7 as a function of oxidation–reduction potential. Examination at various potentials over the range studied showed all the detectable bacteriochlorophyll reactions at 610 nm to be completely reversible, as was found at 615 nm in *Chromatium* D. The E_m of the oxidation–reduction of this bacteriochlorophyll component P600 is about +400 mV. The reversible light-induced absorbance increase measured at 422 nm (Fig. 7) due to P435 has an E_m value similar to that of P600, showing these two spectral components to be associated with the same reaction center bacteriochlorophyll, as was found in *Chromatium* D. The complete disappearance of the P435 reaction (+365 mV to +200 mV) is due to its masking of cytochrome C422 (also reversible) by the opposite absorbance movement, since the dual wavelength spectrophotometer used could not time-resolve the two reactions.

The photooxidation of P600 from +360 mV to 0 mV is attenuated in two steps as the oxidation-reduction potential was lowered. The first is by 50–60% between +360 mV and +200 mV, corresponding with the influx of electrons from cytochrome C422 which became chemically reduced over this range. The second step completes the attenuation during the course of cytochrome C419 reduction and cytochrome C422

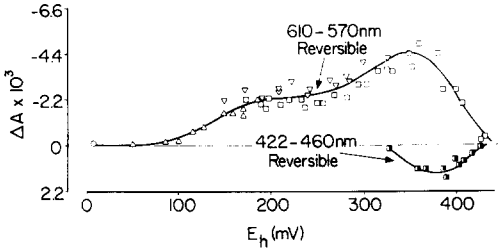

Fig. 7. The photooxidation of bacteriochlorophyll at 610 nm (P600) at 77°K in *R. gelatinosa* as a function of oxidation–reduction potential. Mediators: □ and ◨, B; ▽, C; △, D. Bacteriochlorophyll concentration, 185 μM. Light path 1 mm.

attenuation (see Fig. 9). The final attenuation could be interpreted as being due to the influx of electrons from cytochrome C419 which also displaced those coming in from cytochrome C422. As with *Chromatium* D, attenuation of the P600 reaction results from the reduction of $P600^+$ by the cytochromes at a rate faster than the response time of the spectrophotometer. Hence, since a normal and completed (compare cytochrome C553 and bacteriochlorophyll in *Chromatium* D) theoretical one-electron oxidation-reduction curve is observed for cytochrome C419 and bacteriochlorophyll attenuation, this suggests that the ratio of C419 heme photooxidizable at 77°K and P600 is about 1:1. Furthermore, the ratio of cytochrome C422 to cytochrome C419 is the same (0.5–0.6 to 1) whether it is derived directly from their relative amplitudes of photooxidation (assuming similar extinction coefficients) or by assay of P600 which is independent of their extinction coefficients. The ratio obtained from the P600 titration requires, however, the important consideration that P600 oxidize both cytochromes. Earlier work[18] with *R. gelatinosa* at 77°K showed no alteration in the character of the bacteriochlorophyll light-*minus*-dark difference spectrum from about 585 nm to 620 nm when under oxygen-saturated conditions (light-induced reactions indicating a potential of about +330 mV), or when cytochrome C422 photooxidation was maximal (about +200 mV). This indicates that there was no obvious appearance of a different bacteriochlorophyll species during the titration.

These data strongly suggest that in *R. gelatinosa*, like *Chromatium* D, the same reaction center bacteriochlorophyll oxidizes both the high and low potential cytochromes.

Other work with principal cytochromes of *Chromatium* D[3,21] and *R. rubrum*[12] has shown the high potential cytochromes to be components of the cyclic electron transport system and to be spectrally evident in the light under higher oxidation–reduction potential conditions; the low potential cytochromes dominate at lower potentials and appear to be associated with substrate-linked, non-cyclic electron transport. By analogy with these observations, cytochrome C422 of *R. gelatinosa* would be in cyclic electron transport, and cytochrome C419 in non-cyclic electron transport.

(b) Spectral changes of carotenoids at 77°K

Fig. 8 shows the effect which the oxidation–reduction potential had on the carotenoid photoreactions. All of the visible changes can be ascribed directly to the light-induced reactions of the reaction center bacteriochlorophyll, its acceptor and the cytochrome components with which they interact at 77°K. At oxidation–reduction

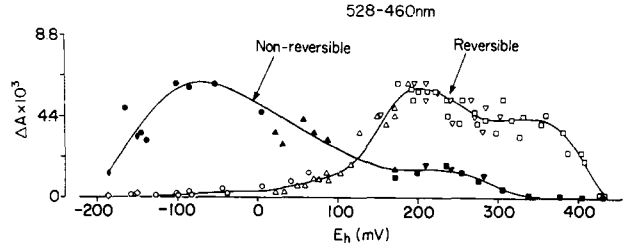

Fig. 8. The photoreactions of carotenoids in *R. gelatinosa* at 77°K as a function of oxidation–reduction potential. Details are given in Fig. 7.

potentials above +450 mV there is no photochemistry due to the chemically oxidized state of all reacting components prior to freezing, nor are there light-induced carotenoid changes. Bacteriochlorophyll photooxidation and carotenoid changes become evident simultaneously as bacteriochlorophyll$^+$ is chemically reduced between the oxidation–reduction potentials of +440 mV and +360 mV. From +330 mV to +200 mV, when cytochrome C422 becomes chemically reduced, the reversible oxidation–reduction of this cytochrome enhances the reversible carotenoid change. Over the same range, a non-reversible carotenoid change is observed corresponding with the non-reversibly oxidized portion of cytochrome C422. The reversible carotenoid change is attenuated over an identical oxidation–reduction potential range to the attenuation of the observed cytochrome C422 and P600. All three attenuation curves fit a theoretical one-electron curve (E_m +135 mV), as shown in Fig. 9. The small amount of the reversible 528 nm change which remains below —100 mV can be associated with the small amount of reversible cytochrome reaction at 422 nm (Fig. 6). As the non-reversible cytochrome C419 photooxidation becomes increasingly dominant, the carotenoid reaction also becomes more non-reversible. Furthermore, as the primary electron acceptor is chemically reduced its final attenuation corresponds well with that of cytochrome C419 photooxidation.

Laser-induced P600 oxidation and 528 nm carotenoid absorbance changes at

+370 mV appear to be the same, each occurring with a rise-time of less than 0.5 μsec and a decay half-time of about 12 msec (experiments with Dr. T. Kihara). At potentials in which cytochrome C419 oxidation is dominant the laser-induced carotenoid change also occurs with a half-time of less than 0.5 μsec; however, this time the reaction

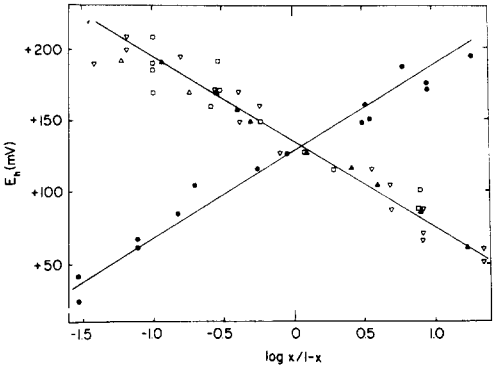

Fig. 9. The course of oxidation–reduction of cytochrome C419, 419–460 nm (●), and the attenuation of the reversible light-induced reactions: cytochrome C422, 422–460 nm (▲); bacteriochlorophyll, 610–570 nm (□); carotenoid, 528–460 nm (▽). In the abscissa for the oxidation–reduction of cytochrome C419, X is the fraction of cytochrome in the oxidized state; for the attenuating reactions X is the fraction attenuated.

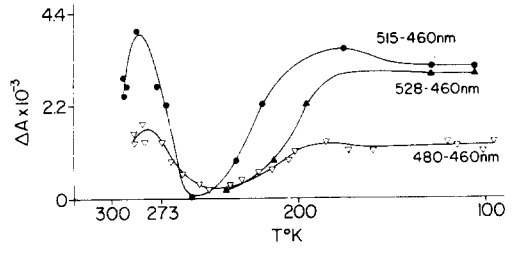

Fig. 10. The effect of temperature on the light-induced reversible carotenoid absorbance changes in *R. gelatinosa*. The effect of temperature was similar during warming or cooling. The ambient oxidation–reduction potential of the chromatophores was estimated from the light-induced reactions to be +200 mV. Bacteriochlorophyll concentration, 200 μM. Light path 1 mm.

is non-reversible. In this case, it would appear that the fast carotenoid change initially follows the bacteriochlorophyll photooxidation. But before there is any significant relaxation due to the return of the electron from the primary electron acceptor ($t_{1/2}$ 12 msec), P600$^+$ is reduced during the non-reversible cytochrome C419 oxidation ($t_{1/2}$ 10–20 μsec). The carotenoid change then becomes permanent at 77°K.

Fig. 10 shows the temperature dependence of the light-induced carotenoid changes. The absorbance changes monitored at various wavelengths characteristic of the carotenoid spectral shifts behave similarly as the temperature is increased from 77°K. The same type of curves are observed as the temperature is decreased from room temperature. The changes are fairly constant between 77°K and 180°K, but at temperatures of about 250°K they are extensively diminished. From 250°K to room temperature the changes reappear with a maximum just above the freezing point. CHANCE AND NISHIMURA[26] described a similar curve between 300°K and 250°K.

TABLE I

MIDPOINT OXIDATION–REDUCTION POTENTIALS OF ELECTRON TRANSFER COMPONENTS

Species	*Component*	*77°K determination* *	*pH*	*Room temperature determination* *	*pH*	*Ref.*
Chromatium D	Primary electron acceptor	−135 mV ($n = 1$)	7.4	−135 mV ($n = 2$) −165 mV ($n = 1$)	7.5 8.0	3 5
	Cytochrome C553	~ +10 mV ($n = 1$)	7.4	+ 10 mV ($n = 1$)	7.0	9
	Cytochrome C555	+325 mV ($n = 1$)	7.4	+330 mV +319 mV ($n = 1$)	7.5	Cited in ref. 3
	Bacteriochlorophyll P600, P435, P400	+470 mV ($n = 1$)	7.4	—		
	Bacteriochlorophyll P890, P800	—		+490 mV ($n = 1$)	7.5	3

* n is the number of electrons transferred.

DISCUSSION

The midpoint potentials of the cytochromes, bacteriochlorophyll, and of the primary electron acceptor of *Chromatium* D determined at 77°K, are listed in Table I and are compared with values determined at room temperature which are taken from the literature. The close similarity of the 77°K and room temperature determinations suggests that the freezing procedure has no significant effect on the measured midpoint potentials of the different components. This means that the light-induced reactions at 77°K are an expression of the oxidation–reduction state of a component at room temperature.

(*a*) *Cytochrome and bacteriochlorophyll reactions*

Evidence has emerged which suggests that one reaction center bacteriochlorophyll complex is responsible for the oxidation of both the high and low potential cytochromes in the photosynthetic bacteria *Chromatium* D (sulphur) and *R. gelatinosa* (non-sulphur). The evidence corroborating the recent analytical and kinetic findings with *Chromatium* D of THORNBERG[27] and PARSON AND CASE[15], respectively, may be summarized as follows. The absorption bands of bacteriochlorophyll at 400 nm, 435 nm and 615 nm in *Chromatium* D appear to be involved in the oxidation of low potential cytochrome C553. Kinetic evidence which supports this finding is presented in the accompanying paper[30]. Since the E_m of the bacteriochlorophyll assayed in the visible spectral region is similar to that found at 808 nm and 883 nm (ref. 3) it appears they are the same reaction center bacteriochlorophyll. Hence, since $P883^+$ is the light generated oxidant for the high potential cytochrome C555[8], it would seem likely that the same bacteriochlorophyll reaction center also serves to oxidize cytochrome C533. Similarly the independent oxidation of cytochromes C419 and C422 in *R. gelatinosa* can be quantitatively related to the reduction of what appears to be the same light-oxidized P600.

In the photosynthetic unit of *Chromatium* D, the evidence suggests that of the 4–7 cytochrome C553 hemes associated with each reaction center bacteriochloro-

phyll[14,27] only two hemes are suitably orientated to the reaction center bacteriochlorophyll to allow electron transfer to occur at 77°K. The two hemes may be parts of one molecule of cytochrome C553 (*cf.* ref. 28) which is directly complexed with reaction center bacteriochlorophyll. Either reduced heme is capable of transferring its electron to bacteriochlorophyll$^+$ at 77°K. If both are reduced, of course only one can become oxidized. Hence at about 0 mV when cytochrome C553 oxidation was just sufficient to reduce the light generated reaction center bacteriochlorophyll$^+$ (*i.e.* each unit contains at least one reduced, light-oxidizable heme), the ratio of the extinction coefficients of cytochrome C553 at 421 nm, and of P600 at 615 nm is about 4. This is expectable if one bacteriochlorophyll$^+$ oxidizes one cytochrome C553 heme considering 70 $mM^{-1} \cdot cm^{-1}$ (per heme) for the oxidized-*minus*-reduced difference of isolated cytochrome C553[28] at 423 nm, and 20 $mM^{-1} \cdot cm^{-1}$ for the absolute absorbance of P600 used by THORNBER[27].

Using 20 $mM^{-1} \cdot cm^{-1}$ for the light-induced change in *Chromatium* D chromatophores at 615 nm indicates that there was one reaction center bacteriochlorophyll (P600) per 120 bulk bacteriochlorophyll molecules. CHANCE *et al.*[29] calculated the ratio of reaction center to bulk bacteriochlorophyll from H^+ uptake measurements in *Chromatium* D to be 1:100. Similar calculations from the light-induced changes at 615 nm at 77°K in the *Chromatium* D subchromatophore preparation (Fraction A) of THORNBER[27] poised at about +350 mV, revealed one reaction center bacteriochlorophyll (P600) per 50 bulk bacteriochlorophyll molecules (P. L. DUTTON AND J. P. THORNBER, unpublished observation); this agrees with the room temperature determinations of THORNBER[27], showing one reaction center bacteriochlorophyll (P883) per 45 bulk bacteriochlorophyll.

Assuming 20 $mM^{-1} \cdot cm^{-1}$ for the light-*minus*-dark difference at 610 nm (P600) from the data obtained from the titrations of *R. gelatinosa*, the extinction coefficients of cytochromes C419 and C422 (per heme) would be about 60 $mM^{-1} \cdot cm^{-1}$. There would be one reaction center bacteriochlorophyll for every 85 bulk bacteriochlorophyll molecules. The molar ratios of bacteriochlorophyll, C419 and C422 (1:1:0.5–0.6) would indicate that about half of the photosynthetic units not contain cytochrome C422. It seems more probable, however, that some of the cytochromes are not light-oxidizable at 77°K, as appears to be the case with the analogous high potential cytochrome C555 in *Chromatium* D. The activation energy of cytochrome C555 is low enough to allow full oxidation at 77°K in a matter of seconds (M. SEIBERT AND D. DEVAULT, personal communication); however, the amplitude of its oxidation diminishes with decreasing temperature, so that it is very small at 77°K (about 5% light-oxidizable).

R. gelatinosa provided the opportunity to observe the control of the high potential cytochrome C422 photooxidation with lowering oxidation–reduction potentials. The course of control described a theoretical one-electron curve, and without further evidence it is difficult to disassociate this curve from the concomitant oxidation-reduction course of the lower potential cytochrome C419. Kinetic studies of cytochrome C419 and C422 photooxidation in *R. gelatinosa* at 77°K[24] show that cytochrome C419 is oxidized with a half-time of 10–20 μsec, which is several times faster than cytochrome C422 oxidation ($t_{1/2}$ approx. 60 μsec). The control of cytochrome C422 oxidation under these conditions does not appear to be a simple matter of a more effective competition by the non-reversibly oxidized cytochrome C419 pool for a limited quantity of reaction center bacteriochlorophyll$^+$, since P600$^+$ was still available throughout the course of

cytochrome C422 attenuation (Figs. 6 and 7). However, if energy or electron transfer between photosynthetic units does not occur at 77°K (it does not appear to occur at room temperature[14,31]), the observed interrelationship of bacteriochlorophyll and cytochromes C419 and C422 can be rationalized. Fig. 11 is a model describing 4 arrangements of electrons in the *R. gelatinosa* photosynthetic unit when poised, for simplicity, at +130 mV before illumination. Each unit, then, represents 25 % of the total

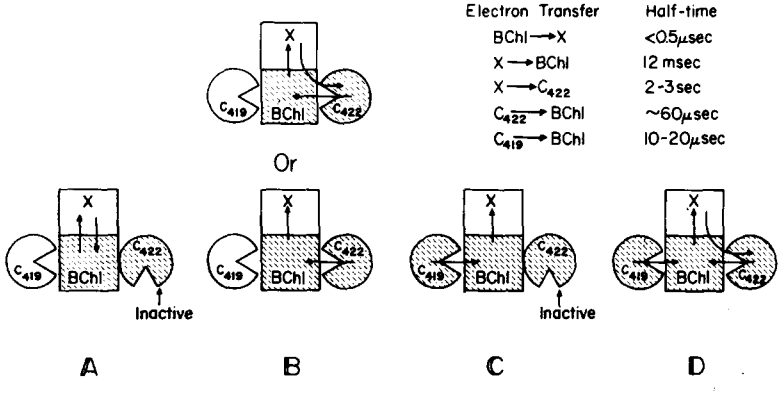

Fig. 11. Representative units of the population of photosynthetic units in chromatophores of *R. gelatinosa* poised at +130 mV, postulated to explain the light-induced reactions at 77°K. Shaded areas are molecules in a reduced state before continuous illumination; the arrows are electron movements during illumination. X represents the primary electron acceptor. The rates of the reactions at 77°K obtained in experiments with Dr. T. Kihara.

population in the chromatophore. When subjected to continuous illumination and assayed as in the experiments, the following effects become evident: in (A) bacteriochlorophyll oxidation; (B) cytochrome C422 oxidation; (C) cytochrome C419 oxidation; and (D) essentially all (about 90 %) cytochrome C419 oxidation. In (D) cytochrome C419 will be oxidized preferentially because: (1) cytochrome C419 reacts about 4 times faster than cytochrome C422; and (2) because over half of the total population of cytochrome C422 reacting is reversibly oxidized (Fig. 11B). Hence if cytochrome C422 is oxidized first, this would allow more than a 50 % opportunity for cytochrome C419, always non-reversibly oxidized, to replace it as the oxidized cytochrome. As observed experimentally in Figs. 6 and 7, the bacteriochlorophyll change is about 25 % of its maximum, the C422 is 50 % attenuated, and the total reduced (50 %) cytochrome C419 pool becomes oxidized.

The small amount of reversible cytochrome C422 oxidation which disappeared from —80 mV to —160 mV may be explained on the same basis, as occurring in a small percentage of units which contain no light-oxidizable cytochrome C419. The similarity of this final attenuation of cytochrome C422 to that of C419 is additional evidence that cytochromes C419 and C422 are using the same reaction center/primary acceptor system.

Fig. 12 summarizes the more clearly defined electron transport reactions operative at low temperatures in *Chromatium* D and *R. gelatinosa*. The *Chromatium* D reactions are fitted into a model for ATP synthesis and NAD^+ reduction. Cytochrome C553 serves as a link by supplying substrate electrons to the cycle in order to replace

those lost to NAD^+ *via* reversed electron flow, although the possibility of direct donation of electrons *via* bacteriochlorophyll to NAD^+ or a lower potential acceptor cannot be ruled out. The oxidation–reduction potential gap between the primary acceptor and the reaction center is, by analogy with sites II and III in mitochondria, sufficient to accomodate two sites of ATP synthesis. *R. gelatinosa* would fit equally well into the same scheme. This basic arrangement of the components which are involved directly with the reaction center bacteriochlorophyll may be more general to bacterial photosynthesis.

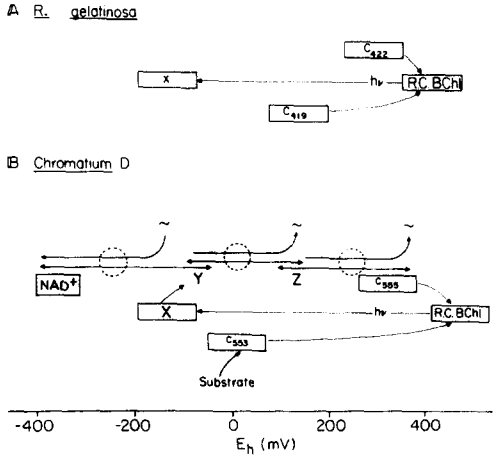

Fig. 12. Summary of the early electron transport reactions in *R. gelatinosa* and *Chromatium* D. The components of known E_m (rectangles) are presented from 10% to 90% oxidized as a function of oxidation–reduction potential. The reactions of *Chromatium* D are fitted into a scheme for energy conservation (~) and NAD^+ reduction. X represents the primary electron acceptor. The dashed circles represent energy transducing electron transport components with midpoint potentials which are energy dependent and which move reversibly (arrows) between electron carriers with non-energy dependent midpoint potentials (*e.g.* Y.Z.C555), after the general mechanism of WILSON AND DUTTON[32]. In this way, energy is transduced for ATP synthesis and reverse electron flow is effected; see also DEVAULT[33], WANG[34] and MITCHELL[35].

(b) Carotenoid absorbance changes

Carotenoids undergo spectral modifications at 77°K which appear to be governed by the oxidation–reduction reactions of the electron transport components engaged in the early phases of photosynthesis. ARNOLD AND CLAYTON[36] observed light-induced changes of bacteriochlorophyll and carotenoids at temperatures as low as 1°K.

While it is not possible to say with any certainty, it does not seem unreasonable to consider that the carotenoid molecules at 77°K in the region of the reaction center unit are responding in some manner to electrostatic field alterations which are caused during the oxidation–reduction reactions. Following the initial formation of bacteriochlorophyll$^+$ and the negatively charged primary acceptor in the light at 77°K, subsequent reactions generate no further net change in charge. The position of the positive charge effectively moves from bacteriochlorophyll$^+$ to the cytochrome as the cytochrome becomes oxidized. The nature of the carotenoid change is descriptive of these changes.

The temperature dependence curve of the carotenoid changes may be interpreted as follows. Below 180°K the carotenoids respond to the oxido–reduction reactions of a

limited number of electron carriers, as described above. At these temperatures the light source used drives the reactions almost to completion since the back reaction rates are relatively slow. Above 180°K other electron transport reactants with high activation energies become involved, the decay rate of the carotenoid signal increases, and the steady state extent of the change becomes correspondingly less. Above 250°K towards physiological temperatures, the carotenoids respond increasingly to other functions associated with the processes of energy conservation[37-39]. At room temperature, however, the fast (less than 50 nsec (T. KIHARA AND D. DEVAULT, personal communication)) carotenoid change which immediately follows a laser flash can still be regarded as responding to bacteriochlorophyll oxidation, since this is much faster than subsequent measured reactions (*e.g.* ion transport), the rates of which are in the millisecond time range[37,39].

ACKNOWLEDGMENTS

The author is indebted to Dr. D. DeVault, Dr. J. P. Thornber, Mr. M. Seibert and Dr. B. Chance for their criticisms and valuable discussions.

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