Primary Oxidation–Reduction Changes during Photosynthesis in *Rhodospirillum rubrum**

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ABSTRACT: The primary electron acceptor in the photosynthetic bacteria *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* has been studied by methods of oxidation–reduction potentiometry combined with instrumentation for measuring photoinduced absorbance changes and photoinduced electron paramagnetic resonance signals. The E_m for the oxidation–reduction dependence of photoinduced changes at pH 7.62 and 25° was found to be −0.022 v for both whole cells of *R. rubrum* and their chromatophores at high ionic strength. Chemically induced absorbance changes, which reproduced the light-induced changes between 740 and 770 mμ, were titrated and found to have an E_m value = −0.06 v at pH 7.65 and 25° for chromatophores of *R. rubrum* at high ionic strength. The location of the absorbance changes suggests that the pigment observed is a porphyrin derivative. The data presented, together with earlier results, are viewed as defining the oxidation–reduction range in which primary events operate in these bacteria. A unified concept of primary oxidation–reduction reactions in bacterial photosynthesis is offered and related to photophosphorylation.

Of the many approaches to studying the phenomena of photosynthesis one of the most rewarding should be that designed to determine the oxidation–reduction range within which the fundamental electron carriers operate. This knowledge is a necessary prerequisite to characterizing and isolating the primary electron donor(s) and acceptor(s). Such information is, of course, essential for a complete understanding of light-energy migration and trapping, and of the secondary chemical events as well.

With the development of sensitive spectrophotometers for measuring light minus dark spectra (Duysens, 1952) and with the application of electron paramagnetic resonance (epr)[1] spectroscopy to biological systems (Commoner *et al.*, 1956; Sogo *et al.*, 1957) part of the tools for conducting a systematic study of early oxidation–reduction changes became available. Since primary events include changes in oxidation state for at least two essential components, it is necessary to control the environmental oxidation–reduction potential as carefully as one ordinarily controls pH, by using appropriate buffers. For the experiments reported herein, techniques of oxidation–reduction potentiometry in the absence of air (Harbury, 1957) were routinely used to establish environmental potentials.

Experiments designed to demonstrate the oxidation–reduction range within which photosynthesis operates have previously met with some success (Loach *et al.*, 1963; Kuntz *et al.*, 1964). In these studies the oxidation–reduction potential of a suspension of chromatophore material was systematically varied, and two characteristic parameters of photosynthetic activity, the light-induced absorbance change and epr signal, were followed. In a continuation of this approach, the following data relate properties of the primary electron acceptor.

Materials and Methods

Rhodospirillum rubrum (No. 1.1.1) and *Rhodopseudomonas spheroides* (No. 2.4.1.C) (both originally supplied by R. Y. Stanier, University of California, Berkeley) were donated by M. Calvin (6/1/63). They have been propagated in modified Hutner's medium (Cohen-Bazire *et al.*, 1957), and used 3–5 days after inoculation from a previous culture. Illumination during growth was provided by banks of fluorescent lights on both sides of 1- or 5-l. culture flasks in a light box at 30°. The intensity of illumination was 1.0×10^4 erg/cm²/sec. Chromatophores were prepared according to procedures previously described (Loach *et al.*, 1963). The reagents methyl viologen, indigotetrasulfonic acid, indigotrisulfonic acid, and indigodisulfonic acid were obtained from K and K Laboratories, Jamaica, N. Y.

All glass apparatus for studies in the absence of air was patterned after those of Harbury (1957) and Loach (Kuntz *et al.*, 1964) with the following modifications. (1) Two rectangular, quartz cuvets having a 1-cm path length with four sides clear and graded seals to Pyrex tubing on each end were obtained from Scientific Glass Apparatus and incorporated into the system. The titration vessel and reference vessel were constructed by

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[1] Abbreviations used: epr, electron paramagnetic resonance; ATP, adenosine triphosphate; NAD^+, nicotinamide-adenine dinucleotide; NADH, reduced NAD^+; ETP, electron transfer particle; EP, elementary particle; FMN, flavin mononucleotide.

TABLE I: Filters and Photomultipliers Used.

Detecting Wavelengths (mμ)	Exciting Wavelengths (mμ)	Photo-multiplier	Detecting Beam[a] Filters[b]	Exciting Beam Filters[b]
		Steady State Measurements		
720–1000	380–620	Dumont 6911	2030 2600 HSB[c]	9780
350–620	720–1000	RCA 6217	9780	2030 2600
		Kinetic Measurements		
427–435	720–1000	RCA C31000	NBP[d] 9780	2030 2600
500–600	720–1000	RCA C31000	3384 9780 4784	2030 2600
783–797	380–620	RCA C70007A	NBP[e] ND[f]	9780
802–816	380–620	RCA C70007A	NBP[g] ND[f]	9780
756–770	380–620	RCA C70007A	NBP[h]	9780

[a] Placed on front of photomultiplier window. [b] All numbers refer to Corning color glasses. A 5-cm water filter was always placed between the exciting light source and the sample. [c] Baird-Atomic high side blocker interference filter (transmits below 880 mμ). [d] Baird-Atomic B-9 narrow band interference filter (transmits 431 ± 4 mμ at band half-width). [e] Baird-Atomic B-9 narrow band interference filter (transmits 790 ± 7 mμ at band half-width). [f] Baird-Atomic 25% transmitting neutral density filter. [g] Baird-Atomic B-9 narrow band interference filter (transmits 809 ± 7 mμ at band half-width). [h] Baird-Atomic B-9 narrow band interference filter (transmits 763 ± 7 mμ at band half-width).

H. S. Martin & Son (Evanston, Ill.). (2) All connections and stopcocks used in the gas train except one are either Delmar (Maywood, Ill.) O-ring units or H. S. Martin O-ring ball joints. A test reductive titration of 1×10^{-6} M flavin mononucleotide (FMN) revealed the system to be quite free from drift over a 6-hr period signifying a very low level of O_2. The inert gas used was research grade argon obtained from Matheson.

Absorption spectra and difference spectra were recorded with a Cary Model 14R spectrophotometer (Applied Physics Corp., Monrovia, Calif.) equipped with a scattered transmission attachment (Model 1462) and an appropriate end window photomultiplier tube as indicated in Table I. An 18-amp tungsten lamp was routinely employed as light source for the detecting beam. The sample compartment was modified to admit a beam of light which illuminated the sample at a right angle to the monochromator beam. The exciting source was a 1000-w GE tungsten projection bulb whose intensity was controlled with a variable transformer. Appropriate filters prevented the exciting beam from reaching the photomultiplier (see Table I).

A device was developed to achieve a scale expansion (beyond that provided by the 0–0.1 absorbance slidewire which was routinely employed for scanning of difference spectra) and an improvement of the signal to noise for measurements in the range 0.0002–0.01 ΔA unit.[2] A gear was matched and meshed with the one which drives the pen on the Cary recorder. The shaft of this gear is that of a precision ten-turn potentiometer which varies the voltage from a standard mercury cell and is in turn applied to the y axis of a Northern Scientific NS-513 Digital Memory oscilloscope (Madison, Wis.). By scanning the spectrum several times it was possible to obtain a 5–10-fold improvement in the signal to noise. The signal was then expanded on an XY recorder (Electro Instruments Model 500, San Diego, Calif.). Data for the reductive titration of FMN at 1×10^{-6} M, for which the absorption at 450 mμ varies from 0.0140 to 0.0005, afforded a precision of better than 2%.

Electron paramagnetic resonance measurements were made with a Varian V-4501 100-kc spectrometer equipped with fieldial and the Northern Scientific Digital Memory oscilloscope mentioned above. Only about a 3–5-fold improvement in signal noise was achieved over what could have been obtained by use of a longer time constant on the Varian unit.

[2] The author especially thanks our Instrument Engineer, R. J. Loyd, for his help and ingenuity in devising electronic gear used in these and other studies to be reported.

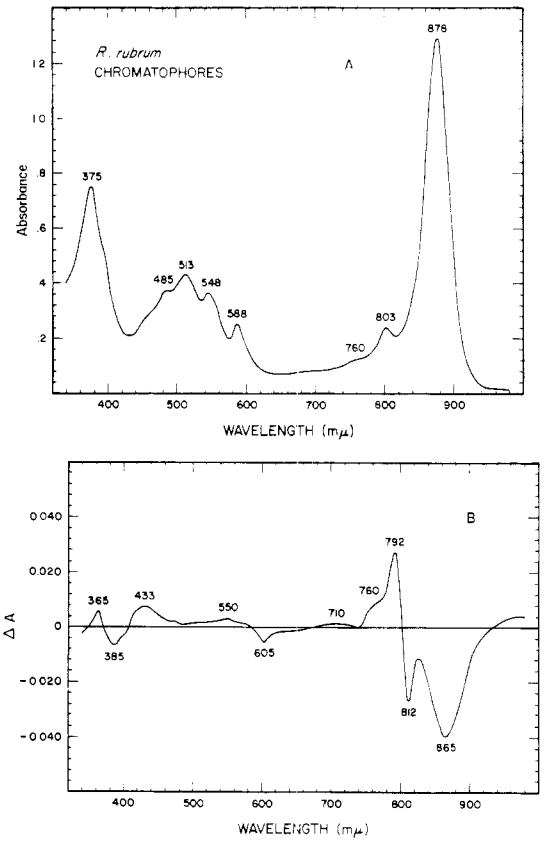

FIGURE 1: Absorbance of chromatophores from *R. rubrum* suspended in 0.05 M phosphate buffer, pH 7.62; 1-cm cuvets. A, absorbance spectrum; B, light-induced absorbance changes. Aerobic conditions; 1×10^{-2} M $K_4Fe(CN)_6$ present. Filter combinations for B are given in Table I. The spectrum of photoinduced changes are very similar to those reported by others (Duysens, 1952; Clayton, 1962a; Kuntz *et al.*, 1964).

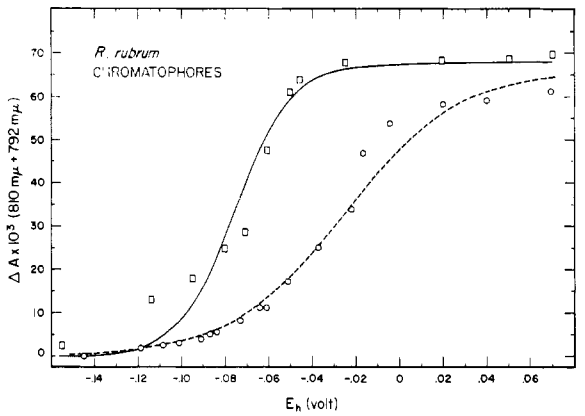

FIGURE 2: Reductive and then oxidative titration of chromatophores from *R. rubrum* suspended in 0.01 M phosphate buffer at pH 6.80. Absorbance at 880 mμ = 1.25. □, experimental points observed during the course of a reductive titration in the presence of 7×10^{-5} M of each indigodisulfonic acid, indigotrisulfonic acid, and indigotetrasulfonic acid; reductant used, $Na_2S_2O_4$ at 0.01 M; the solid line represents a two-electron reduction assuming $E_m = -0.075$ v, 100% oxidation = 0.068 ΔA unit, and 100% reduction = 0 ΔA unit for $E_h = E_m + 2.303RT/n\mathfrak{F}$ log (Ox)/(Red) (Clark, 1960); $t = 25°$. The ordinate represents the sum of the absorbance change at 792 and 810 mμ; since the changes at these two nearby wavelengths are of opposite polarity and presumably both reflect a shift of one absorbance band, a number of errors in measurement can be minimized by taking their sum. O, experimental points for oxidative titration; 0.1 M $K_3Fe(CN)_6$ was the oxidant employed; the suspension of chromatophores was maintained anaerobically overnight (8 hr) after reductive titration. The dashed line represents a theoretical curve for a one-electron oxidation assuming $E_m = -0.025$ v, 100% oxidation = 0.066 ΔA unit, and 100% reduction = 0 ΔA unit for the above equation.

Results

The characteristic absorbance and light-induced absorbance changes of chromatophores from *R. rubrum* are shown in Figure 1. Although the effect on these changes of relatively high environmental potentials (0.3 to 0.5 v) is documented semiquantitatively (Goedheer, 1960; Clayton, 1962a; Loach *et al.*, 1963; Kuntz *et al.*, 1964) those reported to occur in the range of 0 v require considerably more careful measurement. The oxidation–reduction dependence at lower potentials is of particular interest since such data may reflect the chemistry of the primary photoproduced reductant of purple bacteria.

Typical Oxidation–Reduction Experiment. Several titrations are reported in which the photosynthetic material is subjected to a reductive titration followed by an oxidative titration. The details of a typical experiment in which light-induced absorbance changes are measured are as follows.

About 20 ml of a suspension of chromatophores in 0.05 M potassium phosphate buffer at pH 6.80 and 25° is deaerated in all-glass apparatus. The absorbance at 880 mμ for experiments with *R. rubrum* material was between 1.2 and 3.0 for steady-state measurements. The following oxidation–reduction buffers are initially present before air removal: $K_4Fe(CN)_6$, 1×10^{-5} M; 0.5 M $K_2C_2O_4$ and 1×10^{-4} M $FeCl_3$; indigotetrasulfonic acid, 7×10^{-5} M; indigotrisulfonic acid, 7×10^{-5} M; indigodisulfonic acid, 7×10^{-5} M. The use of several buffers in one experiment extends the range through which accurate potentiometric measurements can be made.

Upon removing air from such a system (for vessels used see Kuntz *et al.*, 1964) the cell potential slowly falls through the range +0.4 to +0.3 v where the Fe^{3+}/Fe^{2+} cyanide couple equilibrates with the electrodes. In each experiment conducted in the absence of air an

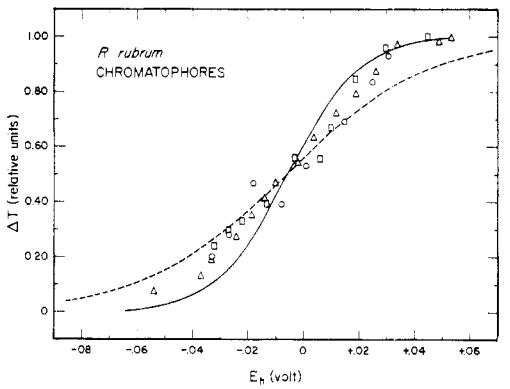

FIGURE 3: Reductive and then oxidative titration of chromatophores from *R. rubrum* suspended in 0.05 M phosphate buffer at pH 7.59; t = 26°. Absorbance at 880 mμ = 2.70; 7×10^{-5} M of each indigotetrasulfonic acid, indigotrisulfonic acid, and indigodisulfonic acid, 0.5 M $K_2C_2O_4$, and 5×10^{-5} M $FeCl_3$ were present initially. Reductant, 0.01 M $Na_2S_2O_4$; oxidant, air. The ordinate is proportional to the change in transmittance (ΔT) which for small changes is also proportional to ΔA. O, points observed at 761 mμ during a reductive titration. △, points observed at 761 mμ during an oxidative titration. □, points observed at 790 mμ during a reductive titration. The solid line represents a theoretical plot for a two-electron oxidation–reduction reaction with E_m = −0.006 v. The dashed line represents a theoretical plot for a one-electron oxidation–reduction reaction with E_m = −0.006 v.

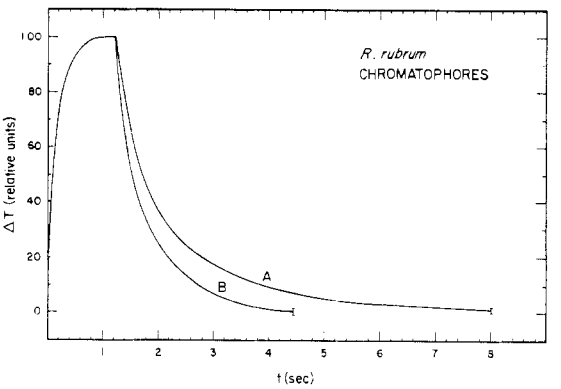

FIGURE 4: Time dependence of absorbance changes at 761 mμ for the experiment shown in Figure 3. Curve A, E_h = +0.0340 v; curve B, E_h = −0.0050 v. The peak-to-peak noise is represented in the figure by the I at one end of the curves. Ten-minutes averaging was employed for each.

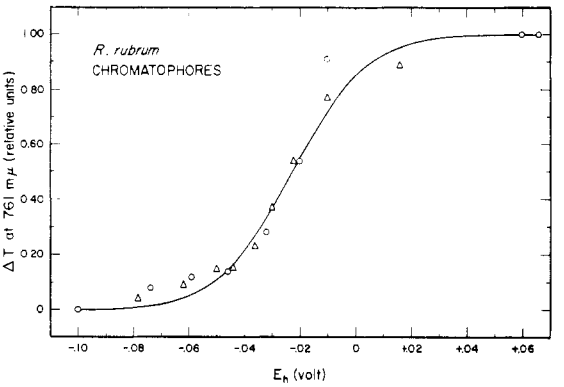

FIGURE 5: Reductive and then oxidative titration of chromatophores from *R. rubrum* suspended in 0.05 M phosphate buffer at pH 7.62. Absorbance at 880 mμ = 1.95; 7×10^{-5} M indigotetrasulfonic acid, indigotrisulfonic acid, and indigodisulfonic acid, 0.5 M $K_2C_2O_4$, and 5×10^{-5} M $FeCl_3$ were present initially. Reductant, 0.01 M $Na_2S_2O_4$; oxidant, air. The ordinate is proportional to the change in transmittance (ΔT) at 761 mμ. O, points observed during a reductive titration. △, points observed during oxidative titration. The solid line represents a theoretical plot for a two-electron oxidation–reduction reaction with E_m = −0.022 v. Dark time between flashes was 30 sec.

endogenous reductant is produced continuously from the chromatophore material, and its presence is detected by the dark reduction of added buffers such as ferricyanide or 2,6-dichlorophenolindophenol. Extensive air removal (3–12 hr) results in a cell potential near 0 v where the indigotetrasulfonic acid couple ($E_{m7} \cong -0.04$ v (Clark, 1960)[3] is beginning to buffer the system. Further lowering of the cell potential cannot be achieved without addition of a reductant such as $Na_2S_2O_4$ or the reduced form of indigodisulfonic acid (E_{m7} = −0.12 v (Clark, 1960)). After addition of a quantity of an appropriate reductant apparent equilibrium is again reached in 3–10 min.

The extent of photoinduced absorbance changes are followed at specific wavelengths of interest at each stage during the reductive experiment. For example the light-induced ΔA (or ΔT) is measured before deaeration, immediately after deaeration, and at each poised potential obtained after extended deaeration and subsequent reductant additions. In this way a record is made of the light-induced signal at specific environmental potentials.

When sufficiently low values of potential are reached so that no photoinduced change can be observed (usually about −0.10 v), the oxidative titration is begun. Typical oxidants used are $K_3Fe(CN)_6$ and air. Distinct increases in cell potential may be achieved in the usual way.

Chromatophores at Low Ionic Strength. During the course of experiments at pH 7 and 25°, it was con-

[3] The terms E_h and E_m are used in this paper after the suggestion of Clark (1960).

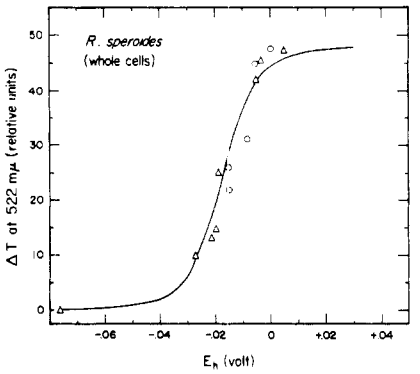

FIGURE 6: Reductive and then oxidative titration of whole cells of *R. spheroides* suspended in 0.05 M phosphate buffer at pH 7.30. Absorbance at 850 mμ = 0.70; 0.1 M $K_2C_2O_4$ and 5×10^{-5} M $FeCl_3$ initially present; 5×10^{-5} M indigotetrasulfonic acid and indigotrisulfonic acid were also present. Reductant, 0.1 M $Na_2S_2O_4$; oxidant, $K_3Fe(CN)_6$. △, points observed during a reductive titration. ○, points observed during oxidative titration. The ordinate is proportional to the change in transmittance (ΔT) at 522 mμ. The solid line represents a theoretical plot for a four-electron oxidation–reduction reaction with $E_m = -0.0180$ v. The exciting light was supplied by Xenon flash lamp (Kemlite S-65-B) which dissipated 90 w-sec energy within 2 msec. Dark time between flashes was 12 sec.

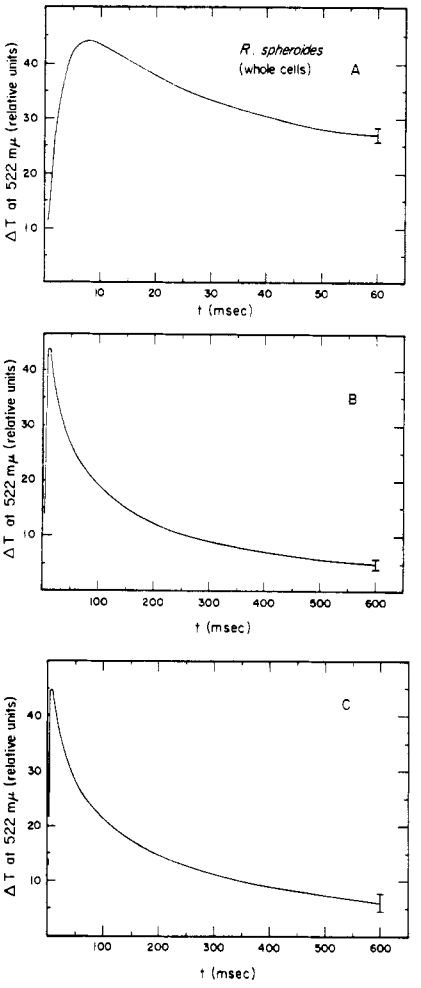

FIGURE 7: Time dependence of absorbance changes at 522 mμ for the experiment shown in Figure 6. A, 80 msec full *x*-axis sweep averaged for 20 min; $E_h = +0.0050$ v (100% oxidized). B, 800 msec full *x*-axis sweep averaged 15 min; $E_h = +0.0050$ v. C, 800 msec full scale averaged for 20 min; $E_h = -0.0205$ v (72% reduced). The peak-to-peak noise is represented in the figure by the I at one end of the curves.

sistently observed that the curve obtained upon reduction was different from that obtained on subsequent oxidation (Figure 2). That is, both the shape of the curve and the point at which 50% of the photoinduced change occurs are different. Such behavior was observed whether the oxidative titration was begun immediately after reduction or whether the system remained in a reduced state overnight (Figure 2) before conducting the oxidative titration. Many experiments in which the photoinduced epr signal was followed also gave results which were inconsistent with a thermodynamically reversible system.

Chromatophores at High Ionic Strength. As was the case for the studies at high potential (Loach *et al.*, 1963), an increase in the ionic strength to values near 1.0 resulted in a system which showed good reversibility. It is not clear why higher ionic strength is effective in allowing oxidation–reduction equilibrium to occur, but in these experiments it may be a result of providing an appropriate carrier (*i.e.*, iron oxalate) which can equilibrate with the membranous pigments of the chromatophore particles. Shown in Figure 3 are the results of an oxidation–reduction titration in which changes in absorbance at 790 and 761 mμ were followed. The data of Figure 3 are not consistent with a one-electron oxidation–reduction reaction and are perhaps more compatible with a two-electron change having a midpoint of −0.006 v. For the results shown in Figure 3, the light which excited the sample was steady light from a 1000-w tungsten projection bulb. The length of the excitation light period varied from 1 to 4 sec followed by 30 sec of dark time. Kinetics of the rise and decay times at 761 mμ are shown in Figure 4. This experiment was conducted with a kinetic spectrometer, the construction of which will be described elsewhere (Loach, manuscript in preparation). It can be seen that the decay rate increases somewhat during reduction. The data of Figure 3 were corrected for this change as described previously (Kuntz *et al.*, 1964).

The decay rates for photochanges in isolated chromatophore systems are relatively slow compared to the corresponding decay in intact cells (compare Figures 4 and 7). For comparison with whole cells (see below) an experiment identical with that reported in Figure 3 was conducted, but the exciting light was supplied by a

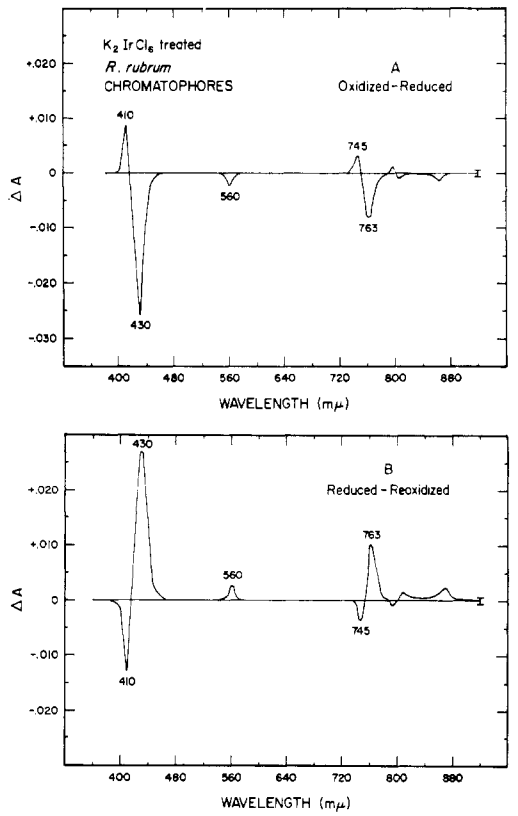

FIGURE 8: K_2IrCl_6 treated chromatophores from *R. rubrum* suspended in 0.05 M phosphate buffer at pH 6.80. Absorbance at 810 mμ = 1.20. *A*, oxidized minus reduced; the oxidized cell formerly had air removed; the reduced form was −0.35 v achieved with $Na_2S_2O_4$. B, reduced minus reoxidized; reduced cell was at E_h = −0.32 v; oxidation was accomplished with a small amount of air. The peak-to-peak noise is represented in the figure by the I at one end of the spectrum.

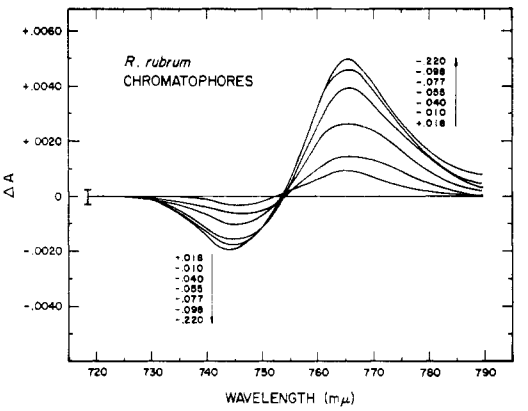

FIGURE 9: Reductive titration showing attendant absorbance changes between 720 and 790 mμ in chromatophores from *R. rubrum* suspended in 0.05 M phosphate buffer, pH 7.65. Absorbance at 803 mμ = 1.10; 0.5 M $K_2C_2O_4$ and 5 × 10^{-5} M $FeCl_3$ initially present. The peak-to-peak noise is represented by I at one end of the scale. Each curve was arbitrarily set equal to zero at 720 mμ; at this wavelength the various curves were between ±0.0010 absorbance unit of the zero line. Reductant used, 0.1 M $Na_2S_2O_4$; potentials at which each curve was recorded are indicated alongside the vericle arrows.

Xenon flash lamp that delivered 90 w-sec of light energy in 2 msec. With this more rapid excitation an appreciable change in absorbance could be observed even with the more rapid decay exhibited by intact cells. These results are shown in Figure 5. The data are in good agreement with those of Figure 3 and fit by a theoretical two-electron reduction. Throughout the experiment the decay kinetics were identical with those of curve A shown in Figure 4.

Other experiments have been performed in which the photoinduced absorbance changes at 433 mμ are followed. Results similar to those shown in Figures 3 and 5 were obtained.

The photoinduced epr signal has been studied in this potential range. Reversible quenching of the photosignal was observed in the same potential range that the photoinduced absorbance changes are quenched. However, these experiments yielded less quantitative results since the data had to be corrected for fractionation of some material out of the electrode vessel. This was due to foaming as a result of the high concentration of chromatophores employed.

Whole Cells. Since it is of the utmost importance to determine whether events in the chromatophores truly represent events in the *in vivo* cell, experiments were conducted with whole cells of *R. rubrum* and *R. spheroides* using several oxidation–reduction buffers (indigotetrasulfonic acid, indigotrisulfonic acid, indigodisulfonic acid, and ferri/ferro oxalate). The cells were obtained by centrifuging and then resuspended in 0.05 M Tris or phosphate buffer. These *in vivo* systems showed rapid response to some of the externally added oxidation–reduction materials and from Figure 6, the quenching of all photochange by lowering the cell E_h is seen to be readily reversible.

The time dependence of the absorbance change at 522 mμ before significant reduction (E_h = +0.0050 v) and at 72% reduction (E_h = −0.0205 v) is shown in Figure 7. There is no significant difference in the decay rates at these two potentials, thus ruling out a change in the steady-state level; 522 mμ, which is one of the many characteristic wavelengths of photoinduced changes in *R. spheroides*, was chosen for this study since the ΔT is large. No significant difference in the E_h dependence for the various wavelengths of photochange (385, 433, 522, 605, 765, 790, 810, and 865 mμ) has been observed in isolated chromatophores.

Although the data of Figures 6 and 7 are for *R. spheroides*, very analogous results were obtained with 4-day-old *R. rubrum* cells for which experiments the photochanges at 433 mμ were followed. That is, the

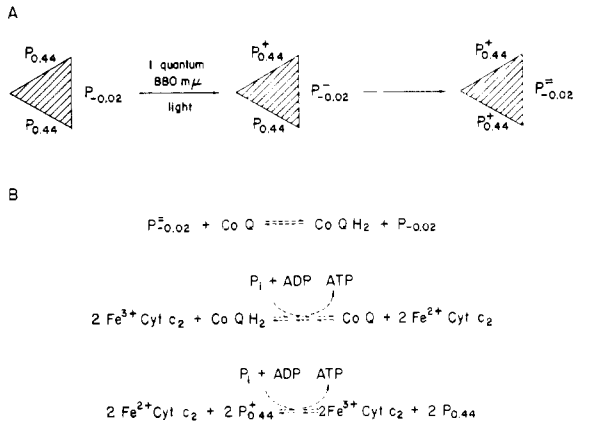

FIGURE 10: Photoinitiated reactions. A, scheme for primary photochemistry in bacterial photosynthesis (for explanation, see text). B, possibly pertinent equations for recombination of the photoproduced electrons and holes. The many arrows and dashed steps allow for participation of a number of unknown reactions.

photochanges were reversibly quenched with a midpoint of −0.025 v.

Chemically Induced Absorbance Changes in Chromatophores. Because the photoinduced changes can be shut off through a characteristic range, the simplest, and perhaps the most likely interpretation is that the primary electron acceptor has been titrated. Such an oxidation–reduction component will almost certainly have absorbance changes associated with its changes in oxidation state although they may be in the ultraviolet. It was previously reported (Kuntz *et al.*, 1964) that no obvious changes in spectra in the wavelength range 400–990 mμ were observed between +0.1 and −0.1 v except for those of a heme complex. However, Figure 8 shows results in this range of potential using more sensitive instrumentation. The reversibility of the changes is indicated by first measuring an oxidized minus reduced spectrum, and then reduced minus reoxidized spectrum over a broad potential range for K_2IrCl_6-treated (Loach *et al.*, 1963) chromatophores from *R. rubrum*. The careful oxidative treatment of chromatophores with K_2IrCl_6 results in a selective irreversible removal of most absorbance above 850 mμ without apparent destruction of the photoactive pigments. There are three regions of importance that can be seen in this figure: (a) changes at 410, 430, and 560 mμ are consistent with those observed for changes between iron(III) and iron(II) in porphyrin complexes; (b) the small changes at 790, 810, and 865 mμ are attributed to a small amount of oxidized primary electron donor (Kuntz *et al.*, 1964); (c) the newly observed changes at 745 and 763 mμ suggest a "bacteriochlorophyll type" of complex which can undergo a reversible oxidation–reduction change.

Similar changes were observed with untreated chromatophores. The potential range for the changes is indicated in Figure 9. The chemically induced change at 763 mμ is also seen as a response to light absorbance in all chromatophore systems studied (*R. rubrum*, *R. spheroides*, and *Chromatium*). This absorbance change can be produced to the same extent (to within 10%) either by light excitation or by chemical reduction. The change at 763 mμ may be associated with a change in the opposite direction at 745 mμ. Preliminary results indicate that no epr signal either accompanies reduction or is a transient during reduction.

Discussion

For the interpretation of the results the equilibria shown below might be considered. For this discussion

$$\text{buffer OX} + P_1P_2^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_1P_2 + \text{buffer red}$$

$$k_2 \uparrow\downarrow k_{-2}$$

$$P_1^+ \quad P_2^-$$

only, P_1 represents the primary electron donor, P_2 the primary electron acceptor, and it is assumed that only P_1P_2 is photoactive. Two extreme cases may be considered. (a) If the rate of formation of P_1P_2 by the reaction with the rate constant k_1 is much faster than the light-driven reaction with rate constant k_2, no dependence on E_h would be observed, provided a wavelength characteristic of P_1 oxidation–reduction change was being followed; that is, as quickly as part of the dark equilibrium concentration of P_1P_2 was converted to $P_1^+P_2^-$ by light, more P_1P_2 would be regenerated until all P_1 is converted to P_1^+ (assuming saturating light intensity). On the other hand, if a wavelength of photochange is followed which is characteristic of P_2, only that amount of change would be observed which reflects directly the dark equilibrium concentration of P_1P_2. (b) For the second extreme case, if the light-driven reaction with rate constant k_2 is much faster than the dark reaction with rate constant k_1, then following a wavelength characteristic of either P_1 or P_2 changes should give the same results and reflect the true dark equilibrium concentration of P_1P_2. Since the experiment shown in Figure 5 (fast excitation) gave results very similar to those shown in Figure 3 (slower excitation), the chromatophore systems fall under case (b) rather than in case (a), or somewhere in between the extremes. Also consistent with case (b) is the similarity of the E_h dependence at all wavelengths thus far examined, that is, 433, 522, 761, 790, 810, and 865 mμ.

Figures 3, 5, and 6 present data describing a fundamental property of the primary electron acceptor molecule in bacterial photosynthesis of the type found in *R. rubrum* and *R. spheroides*; that is, its E_m value for change in oxidation state is −0.02 v at pH 7.6 and 26°. Thus the results together with earlier data (Loach *et al.*, 1963; Kuntz *et al.*, 1964) may be summarized by the scheme shown in Figure 10. $P_{0.44}$ and $P_{-0.02}$ are used to

represent the respective primary electron donor and acceptor molecules. This kind of abbreviation is used to stress the midpoint potential of the oxidation–reduction couple displayed by the pigment and to stress a lack of knowledge about its chemical composition, other than the fact that $P_{0.44}$ is probably a porphyrin derivative. The plus and minus superscripts are used merely to keep in mind that oxidation and reduction have occurred. They are not intended to indicate additional knowledge about the primary species. The cross-hatched triangle represents the key part of the photosynthetic unit (about which little is known) which allows a quantum of light energy to cause a primary oxidation and reduction, but also poses sufficient barrier toward back interaction that oxidation–reduction reactions useful to the cell may ensue more rapidly.

A unique feature of the scheme is the suggestion that 1 quantum of 880-mμ light may have the net result of the removal of two electrons, one from each of two $P_{0.44}$ molecules, in order to account for the reduction of $P_{-0.02}$. Consistent with this assignment are the data of Vredenberg and Duysens (1964) who found the quantum requirement for cytochrome oxidation to be significantly less than one. They also showed by low-temperature studies that cytochrome oxidation is probably not a primary event, but a dark reaction perhaps as indicated by the dashed arrows of Figure 10. Vrendenberg and Duysens assumed the specific extinctions assigned to the cytochromes studied were too low and that the actual quantum requirement is close to one. However, their data are better fit by a minimum quantum requirement of 0.5 which would follow from a scheme such as that presented in Figure 10. Consistent with a two-electron reduction is the lack of identification of an epr signal during dark reduction.

Several alternate assignments would presently be consistent within the limitation of the data. For example, the primary photochemical event might be a one-electron oxidation–reduction reaction after which the primary electron acceptor molecule rapidly picks up a second electron from its environment. This is, of course, equivalent to the scheme pictured in Figure 10 if the second electron is extracted from a nearby "special bacteriochlorophyll" molecule. Other electron donors are in ample supply in the growth media of these bacteria. There are many ways of testing the various possibilities, but each depends on a more exact knowledge of the primary electron donor and acceptor molecules.

In support of the description of primary events reported herein is the concomitant dependence of adenosine triphosphate (ATP) formation on the oxidation–reduction level of chromatophore systems (Horio and Kamen, 1962; Bose and Gest, 1963a).[4] The establishment of an environment sufficiently electronegative (excess ascorbate and a mediator) to reduce cytochrome *b* ($E_m \cong -0.03$ v at pH 7.0) resulted in a cessation of ATP formation. Also reflecting the significance of the primary electron acceptor is the "overreduction" by high concentrations (2×10^{-3} M) of nicotinamide–adenine dinucleotide (NADH) in the presence of the mediator methyl or benzyl viologen (Bose and Gest, 1963a; Nozaki *et al.*, 1963). "Overreduction" could also be accomplished by H_2 in the presence of a hydrogenase to which methyl or benzyl viologen was also added. The role played by $P_{-0.02}$ is apparent in each case.

The rate with which ATP is formed was also found to be diminished at higher potentials (Horio and Kamen, 1962) although the potentials were not always sufficiently high to account for oxidation of $P_{0.44}$. It may be noted that considerably slower decay rates for the light-induced absorbance change were observed in the present study (especially for whole cells) as the environmental potential increased through the range 0 to 0.2 v. This change in decay rate has also been described by others (Heise and Vernon, 1963; Kuntz *et al.*, 1964). Perhaps the less rapid restoration of $P_{0.44}^+$ to its normal dark form explains the lower rates of ATP formation.

A most interesting aspect associated with bacterial photosynthesis is the light-dependent reduction of NAD^+ (Frenkel, 1961; Vernon and Ash, 1959) and the so-called noncyclic photophosphorylation (Nozaki *et al.*, 1963) which can accompany net NAD^+ reduction. A quantum requirement of 2–3 found by Amesz (1963) for reduction of one NAD^+ would require that a major part (25% for a quantum act with a yield = 0.5 or 50% for a quantum act with a yield = 1.0) of the energy released in the 0.5-v span of back interaction between $P_{-0.02}^{2-}$ to $P_{0.44}^+$ would have to be coupled in order spontaneously to reduce NAD^+ from substrates whose E_m values are normally near 0 v (succinate, $E_{m7.6} \sim 0.03$ v (Clark, 1960); ascorbate, $E_{m7.6} \sim 0.05$ v (Ball, 1937). Succinate represents one of the poorer reductants which is effective, but presumably only in the presence of sucrose (Amesz, 1963). The kinetics for NAD^+ reduction in *R. rubrum* reported by Amesz show a definite lag period of several seconds observed by both ΔA and fluorescence measurements after the onset of illumination. The lag of several seconds for NAD^+ reduction is consistent with the occurrence of several coupled reactions but not with a role where NAD^+ is on a more direct path of electron flow from a primary light-reduced species. Similarities between mitochondrial systems and photosynthetic units are clearly abundant. Perhaps the reduction of NAD^+ in chromatophore systems is yet one more example of a similar phenomenon observed in mitochondrial systems and referred to as an energy-linked function (Energy-Linked Functions of Mitochondria, 1963). In particular the suggestions of Bose and Gest (1963b) and Stanier (1961) are pertinent.

The chemically induced changes at 763 mμ, having an E_m value = −0.060 v for chromatophores from *R. rubrum* (pH 7.65 and 25°), seem to be distinct from those that might be exhibited by the primary electron acceptor molecule whose E_m under identical conditions

[4] The chromatophore preparations used in these studies display photophosphorylating ability to approximately the same extent as those systems studied by Horio and Kamen, 1962; Bose and Gest, 1963a,b.

is −0.022 v. Absorbance changes associated with $P_{-0.02}$ have not yet been observed.

The ultimate understanding of photosynthesis in any single organism will undoubtedly be the result of the synthesis of studies from many laboratories each focused on different steps of complexity from whole cells to purified components. For the study of primary events, unmasked by many subsequent reactions, the preparation of material corresponding to the electron transfer particle (ETP) (Green, 1962) or elementary particle (EP) (Fernández-Morán, *et al.*, 1964) of mitochondria should contribute markedly. Evidence presented in this paper may be viewed as promoting some additional confidence that the primary pigments will maintain their integrity through admittedly "crude" cell disrupting devices and oxidative treatment.

References

Amesz, J. (1963), *Biochim. Biophys. Acta 66*, 3881.

Ball, E. G. (1937), *J. Biol. Chem. 118*, 219.

Bose, S. K., and Gest, H. (1963a), *Proc. Natl. Acad. Sci. U. S. 49*, 337.

Bose, S. K., and Gest, H. (1963b), *in* Energy-Linked Functions of Mitochondria, Chance, B., Ed., 1st Colloquium of Johnson Research Foundation, New York, N. Y., Academic.

Chance, B., Ed., (1963), Energy-Linked Functions of Mitochondria, 1st Colloquium of Johnson Research Foundation, New York, N. Y., Academic.

Clark, W. M. (1960), Oxidation-Reduction Potentials of Organic Systems, Baltimore, Md., Williams and Williams.

Clayton, R. K. (1962a), *Photochem. Photobiol. 1*, 201.

Clayton, R. K., (1962b), *Photochem. Photobiol. 1*, 305.

Cohen-Bazire, G., Sistrom, W. R., and Stanier, R. Y. (1957), *J. Cellular Comp. Physiol. 49*, 25.

Commoner, B., Heise, J. J., and Townsend, J. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 710.

Duysens, L. N. M. (1952), Ph.D. Thesis, Utrecht.

Fernández-Morán, H., Oda, T., Blair, P. V., and Green, D. E. (1964), *J. Cell Biol. 22*, 63.

Frenkel, A. W. (1961), *in* A Symposium on Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., John Hopkins Press, p. 587.

Goedheer. J. C. (1960), *Biochim. Biophys. Acta 38*, 389.

Green, D. E. (1962), *Comp. Biochem. Physiol. 4*, 81.

Harbury, H. A. (1957), *J. Biol. Chem. 225*, 1009.

Heise, J. J., and Vernon, L. P. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro A., and Vernon, L. P., Ed., Yellow Springs, Ohio, Antioch Press, p 343.

Horio, T., and Kamen, M. D. (1962), *Biochemistry 1*, 144.

Kuntz, Jr., I D., Loach, P. A., and Calvin, M. (1964), *Biophys. J. 4*, 227.

Loach, P. A., Androes, G. M., Maksim, A. F., and Calvin, M. (1963), *Photochem. Photobiol. 2*, 443.

Loach, P. A., and Calvin, M. (1963), *Biochemistry 2*, 361.

Loach, P. A., and Calvin, M. (1964), *Nature 202*, 343.

Nozaki, M., Tagawa, K., and Arnon, D. I. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro, A., and Vernon, L. P., Ed., Yellow Springs, Ohio, Antioch Press, p 175.

Sogo, P. B., Pon, N. G., and Calvin, M. (1957), *Proc. Natl. Acad. Sci. U. S. 43*, 387.

Stanier, R. Y. (1961), *Bacteriol. Rev. 25*, 1.

Vernon, L. P., and Ash, O. K. (1959), *J. Biol. Chem. 234*, 1878.

Vredenberg, W. J., and Duysens, L. N. M. (1964), *Biochim. Biophys. Acta 79*, 456.