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Primary charge separation in bacterial photosynthesis: Oxidized chlorophylls and reduced pheophytin

(reduced bacteriopheophytin/transient electron acceptor)

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ABSTRACT **Bacteriopheophytin, the magnesium-free base of bacteriochlorophyll, undergoes reversible one-electron reduction in organic solvents to yield an anionic free radical with characteristic optical and electron spin resonance spectra. The reduction potential of bacteriopheophytin, $E_{1/2} \approx -0.55$ V against a normal hydrogen electrode, compared to $E_{1/2} \approx -0.85$ V for bacteriochlorophyll, renders it a likely electron acceptor in the primary charge separation of photosynthesis. Comparison of these data with picosecond optical changes recently observed upon pulsed laser excitation of bacterial reaction centers leads us to propose that bacteriopheophytin is indeed a transient electron acceptor and that the primary charge separation of bacterial photosynthesis occurs between the bacteriochlorophyll complex P_{870} and bacteriopheophytin to yield the radicals of the oxidized chlorophyll dimer cation and reduced pheophytin anion.**

Light is converted by green plants and photosynthetic bacteria into chemical energy by the creation of an oxidant (P^+) and a reductant (X^-) which result from ejection of an electron from (bacterio)chlorophyll (P) to a nearby acceptor (X). An extensive body of evidence (for reviews, see refs. 1–5) leads to the conclusion that light impinging on a photosynthetic organism is gathered by antenna chlorophylls (and other pigments) and funneled to a special environment, the reaction center, wherein a chlorophyll "special pair" (P_{700} in plants and P_{870} in purple bacteria) undergoes one electron oxidation to yield a π cation radical [P_{700}^+ and P_{870}^+ (5)] with characteristic optical, electron spin resonance (ESR), electron-nuclear double resonance, and redox properties. Isolation of photochemically active reaction centers of purple bacteria simplifies the study of the charge separation since each reaction center contains only three different polypeptides (about 70,000 daltons), four bacteriochlorophylls, two bacteriopheophytins, one ubiquinone, and a nonheme iron, and is free of cytochromes, copper, and antenna bacteriochlorophyll. Circular dichroism (CD) spectra indicate that, within the reaction center, the bacteriochlorophylls (BChls) are strongly exciton coupled, with weaker interactions with the bacteriopheophytins (BPhs). The creation of P^+_{870}, on oxidation of the reaction center, disrupts the CD interactions and the orientation of the pigment molecules is disturbed (1–3).

The nature of the electron acceptor (X) is less clear (1–4). Midpoint potentials, E_m, for the one electron reduction of X in different species of bacteria range between 0 and −150 mv [against a normal hydrogen electrode (NHE)], but values as low as −350 mv have been reported (2, 6, 7). Removal of ubiquinone (UQ) from *Rhodopseudomonas spheroides* reaction centers inhibits photochemical activity (8). To reconcile the UQ data with ESR evidence of an iron compound, a complex of iron–ubiquinone (Fe–UQ), $E_m = -50$ mv, is generally considered to be a primary electron acceptor (9–11).

Recent nanosecond and picosecond laser excitations of *R. spheroides* reaction centers, poised at potentials where X(Fe–UQ) is reduced, reveal a transient state, P^F, which forms with high quantum yield. P^F exhibits optical changes that implicate both BChl and BPh and decays with an exponential time $\tau = 150$–250 psec with the concomitant appearance of $P^+_{870}X^-$ when X is not initially reduced. Strikingly, P_{870} oxidation seems to occur with X reduced as well (12–15). We present here evidence that equates P^F with the primary charge separation of bacterial photosynthesis and propose that bacteriopheophytin is the transient electron acceptor of the P_{870} oxidation.

Abbreviations: BPh, bacteriopheophytin; BPh^-, reduced BPh; BChl, bacteriochlorophyll; ESR, electron spin resonance; $E_{1/2}$, half wave potential; E_m, midpoint potential; P_{870}, BChl special pair with 870 nm absorbance; P^*_{870}, excited singlet of P_{870}; P^+_{870}, oxidized P_{870}; NHE, normal hydrogen electrode; UQ, ubiquinone; X, electron acceptor, iron-ubiquinone complex.

EXPERIMENTAL

Cyclic voltammetry, controlled potential electrolysis, and coulometry were performed using a Princeton Applied Research potentiostat 173 equipped with a function generator 175 and a digital coulometer 179. Potentials were determined on 10^{-3} M solutions containing 0.1 M tetrapropylammonium perchlorate against an aqueous saturated calomel electrode (= NHE −0.24 V). Optical spectra were obtained with a vacuum electrolysis cell on a Cary 17 spectrophotometer. The optical data used for the difference spectra of Figs. 3 and 4 were digitized using a Vanguard scanner and processed on a Control Data Corp. 6600 computer. ESR spectra were collected at X-band on a Varian E-12 spectrometer, equipped with a Field/Frequency Lock accessory and ramped by an SDS Sigma 2 computer. All ESR and optical spectra were obtained on samples prepared on a vacuum line using dried, distilled, and outgassed solvents with oxygen rigorously excluded. The chemical and electrochemical techniques have been described (16–18). A Coherent Radiation krypton ion laser and a quartz halogen Oriel lamp were used for the photochemical reductions. BPh *a* was prepared from BChl *a* extracted from *Chromatium vinosum* [BChls found in *C. vinosum* and *R. spheroides* have the same chemical composition (19)]. BPh was dissolved in CH_2Cl_2, which was pumped away to codistill water, and the pigment was dried under reduced pressure (10^{-3} Pa) for several hours.

RESULTS

The anion radical of bacteriopheophytin

BPh exhibits a reversible, one electron, halfwave reduction potential $E_{1/2}$ of −0.58 against NHE in CH_2Cl_2. Electro-

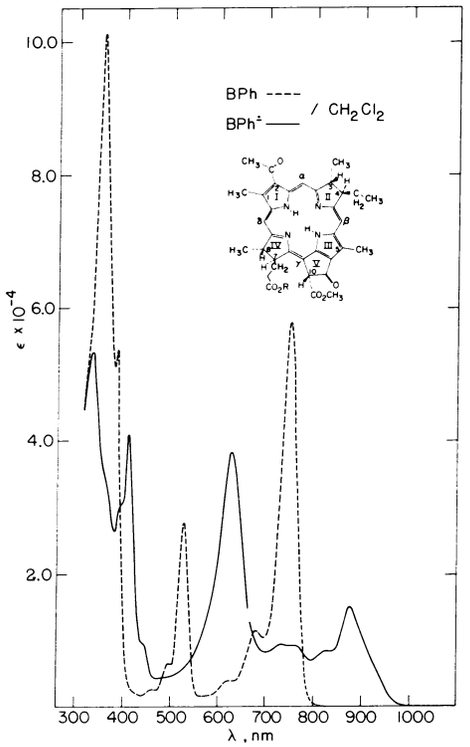

FIG. 1. Optical spectra of BPh (- - -) and of the anion radical BPh^- (—) in CH_2Cl_2.

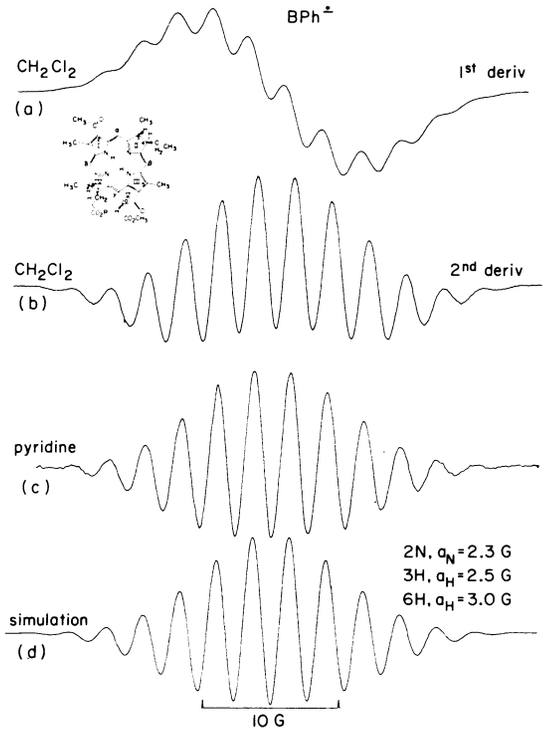

FIG. 2. (a) First and (b) second derivative ESR spectra of BPh^- in CH_2Cl_2, (c) second derivative spectrum of BPh^- in pyridine, and (d) computer simulation of (c) using the splitting constants shown.

chemical reduction requires one electron and yields the optical spectra shown in Fig. 1. One electron oxidation regenerates better than 95% of the original BPh. The system can be recycled to again yield the radical spectrum. The ESR spectrum of BPh^- in CH_2Cl_2 at room temperature exhibits a g-value of 2.0030 (±0.0002) and a 12 line, partially resolved hyperfine structure (Fig. 2)*. Similar spectra, with only minor variations in apparent splitting constants and linewidths are obtained by chemical reduction with an 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) ether/potassium complex in 2-methyltetrahydrofuran (20, 21) and by photochemical reduction in pyridine in the presence of Na_2S (22).

The ESR spectrum in pyridine is displayed for comparison with a computer simulation (Fig. 2). The simulation assumes interaction of the unpaired electron spin density with the following nuclei: (*a*) 2 nitrogens (rings II and IV) with hyperfine splitting constant a_N = 2.3 G (2.3 × 10^{-4} tesla), (*b*) 3 protons, average a_H = 2.5 G for the hydrogens of position δ, α, and β, and (*c*) 6 protons, average $a^H_{CH_3}$ = 3.0 G for the methyl groups 1 and 5 on rings I and III, respectively. These assignments are deduced from ESR and electron-nuclear double resonance observations on BPh^-, deuterated BPh^-, and selectively deuterated BPh^- †. In addition, they are in accord with the ESR spectra of the anion radical of a model compound, free base tetraphenyl bacteriochlorin, also selectively deuterated, and all the above ESR data are in general agreement with Pariser, Parr, Pople self-consistent-field molecular orbital calculations by Felton‡. Thus, positions 1, 2, 5, 6, δ, α, and β each carry about 0.1 unit of unpaired spin while the spin density in rings II and IV is concentrated in the nitrogens.

The good agreement with model systems and molecular orbital calculations minimizes the possibility that the species observed is the result of a proton addition: $BPh^- + H^+ \rightarrow$ BPhH·. Molecular orbital calculations for BPhH· indicate that large spin densities would exist at the site adjacent to the proton addition (presumably one of the *meso* positions) and would result in large couplings with the added proton‡. We observe no initial difference in the ESR spectrum of BPh^- prepared photochemically in pyridine that contains small amounts of H_2O or D_2O (the radical eventually undergoes isotopic exchange, *not* addition) and conversely, we detect no difference in the ESR spectra of perdeutero BPh^- in perdeutero pyridine containing D_2O or H_2O.

In conclusion, we find an anion radical of BPh stable in several solvents with a halfwave reduction potential which varies slightly from solvent to solvent: $E_{1/2}$ (against NHE) = −0.58 V in CH_2Cl_2, −0.54 in butyronitrile, −0.51 in dimethylformamide, and −0.52 in ethanol [pH = 9.4 (23)] for an averaged $E_{1/2} \simeq -0.55$ V. These compare with $E_{1/2}$ values of +0.64 and −0.86 V for oxidation and reduction of BChl in CH_2Cl_2.

A biological role for bacteriopheophytin

We now propose that, in bacterial reaction centers, bacteriopheophytin acts as a transient electron acceptor on a picosecond time scale and that the electron donor is the P_{870} special pair. The primary charge separation of bacterial photosyn-

* The spectrum collapses to a singlet, ΔH_{ptp} = 13 G at 100 K.

† The different nuclear spin of deuterium, I = 1 compared with I = ½ for hydrogen, combined with the difference in gyromagnetic ratio yield large and predictable ESR changes on substitution of deuterium for hydrogen. See ref. 33 for examples.

‡ J. Fajer, A. Forman, M. S. Davis, L. D. Spaulding, D. C. Brune, and R. H. Felton, submitted.

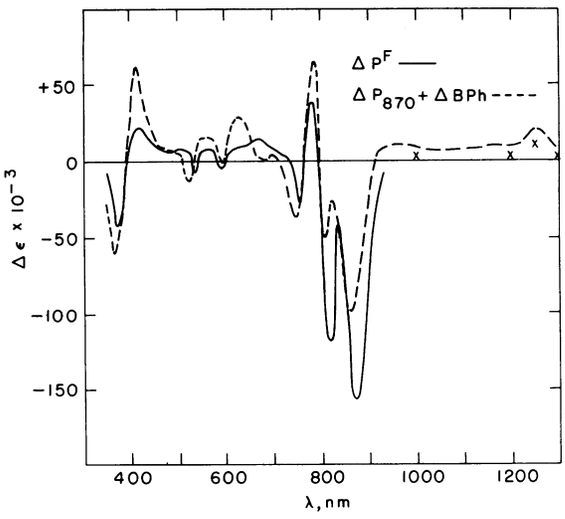

FIG. 3. Comparison of the laser induced optical changes, ΔP^F, with the changes calculated on the assumption that $\Delta P^F = \Delta P_{870} + \Delta BPh$, the changes caused by the oxidation of P_{870} and the reduction of BPh. ΔP^F observed (— and points ×); ΔP^F calculated (- - - -).

thesis is thus the photochemical creation of the radicals of the cation P^+_{870} and the anion BPh^- §. We examine here the reasons for and the consequences of this suggestion in terms of the existing experimental data and energetics involved.

The idea that the transient P^F is a pure excited singlet has been considered and discarded (13, 14) because of the order of magnitude discrepancy between the lifetime of the singlet estimated from fluorescence measurements (24, 25) and that of P^F. A triplet state has been observed *in vivo*, but only when the electron acceptor X is reduced (26–28), and it probably forms (2, 12) from P^F (see below).

The oxidation of P_{870} is well characterized by steady-state photochemical and chemical techniques and leads to typical difference spectra (1, 2). These reflect the disruption of the complex of two sets of BChls that give rise to the 800- and 870-nm bands and the appearance of the π cation radical (30–32) of the bacteriochlorophyll pair, P_{870}^+. Of all the radicals possibly involved in photosynthesis, i.e., $BChl^+$, $BChl^-$, BPh^+, BPh^-, and P_{870}^+, that have been observed optically (17, 33–35), *only* the dimer absorbs in the red region between 1100 and 1300 nm (λ_{max} = 1250 nm). The most recent psec data indicate (15) that the 1250-nm band appears immediately upon excitation. The rapid bleaching of the 870-nm band ($\leq$7 psec) and the quantum yield of about 1 found for the bleaching of the 870-nm band upon 880-nm excitation, even at 4 K (29; R. K. Clayton, private communication) further suggest that oxidation of the P_{870} complex is a primary step which proceeds from the excited singlet.

If we thus accept that oxidized P_{870} is the electron donor observed immediately upon psec excitation and if X (the UQ–Fe complex) is already reduced, then the choice of primary electron acceptor is limited to either BChl or BPh.

If it is BPh, then P^F represents the sum of the oxidized P_{870} and the reduced BPh, and the optical changes reported for the appearance of P^F, ΔP^F, are given by the spectral differences $\Delta P_{870} + \Delta BPh$ where $\Delta P_{870} = P^+_{870} - P_{870}$ and $\Delta BPh = BPh^- - BPh$. ΔP_{870} has been determined both by photo- and chemically-induced oxidation. The data of Reed (34) have been used to calculate Fig. 3. [The absorption due to X and X^- is small (35, 36) relative to the other changes in P_{870}, so we feel justified in ignoring it. Use of a chemically oxidized ΔP_{870} spectrum (35) yields results similar to those displayed in Fig. 3.] ΔP^F is a composite of the data reported by Parson *et al.* for nsec excitation with X reduced (Fig. 2A of ref. 13) for the region 300–700 nm and the psec data with X not reduced, for the 700- to 900-region (Fig. 1 of ref. 13). The first part of the spectrum is consistent with the sparser data obtained after 20 psec in the 400- to 700-region with X not reduced (13). Kaufmann *et al.* (14) reported similar results. The points X shown in the 1000- to 1300-nm region are the data of Dutton *et al.* (15), which were obtained under nonsaturating light intensities.

The discrepancy near 800 nm in the calculated spectrum is due in part to the bleaching of that band as it undergoes kinetics of its own with $\tau \simeq 50$ psec (13). These changes may well reflect (13) the disruption of the reaction center when P^+_{870} is formed and would be consistent with circular dichroism data (3). At large changes in absorbance (ΔA), the psec data also contain apparent artifacts such as $\Delta A = 0.16$ for a solution that has a nominal absorbance $A = 0.12$ at 870 nm (see Fig. 1 of ref. 13). We emphasize the good agreement between practically all maxima and minima in the calculated and experimental spectra over the entire 350- to 1300-nm region.

According to the mechanism we propose, the optical changes should first reflect the appearance of P^+_{870} and BPh^-. Then, if X was not originally reduced, the disappearance of BPh^- should reveal the spectrum of P^+_{870} and X^-, i.e., the standard $P^+X^- - PX$ difference spectrum:

$$P^*_{870} \longrightarrow P^+_{870} + BPh^- + X \longrightarrow P^+_{870} + X^- + BPh$$

Experimental data at several wavelengths typical of both P_{870} and BPh confirm this suggestion (12–15). Furthermore, since BPh^- does not absorb above 950 nm (see Fig. 1), the infrared bands characteristic of P^+_{870} should appear immediately and undergo no further kinetics until P^+_{870} is reduced again. This is also observed (15). The experimental data, kinetic as well as optical, thus support our scheme.

Note that if X is initially reduced, P^F can decay via a back reaction to yield P^*_{870} and fluorescence or a triplet of P_{870}. Increased fluorescence (37) and triplets consonant with the P_{870} pair but with unusual spin polarization have been observed (26, 28, 38, 40) when X is reduced¶. The triplet is probably the state P^R detected by Parson *et al.* (12).

We consider now the energetics of the reaction we postulated:

$$P_{870} + BPh \xrightarrow[870\,nm]{h\nu} P^+_{870} + BPh^-$$

The midpoint potential for the one electron oxidation of P_{870} is +0.45 V against NHE (2, 3, 41). $E_{1/2}$ of a reversible, one-electron process virtually equals the thermodynamic potential, and therefore the $E_{1/2}$ values found for the BPh reduction can be used to estimate the free energy gained by the photochemically-induced charge separation. $E_{1/2} \simeq$

§ BPh presumably occurs in intact organisms as well as in isolated reaction centers.

¶ Recombination of the transient radicals of P^F with a dynamic electron polarization mechanism could induce the unusual spin population of the triplet (39). The $BPh^- - P^+_{870}$ annihilation was recently considered by Thurnauer *et al.* (40). See also Dutton *et al.* (15).

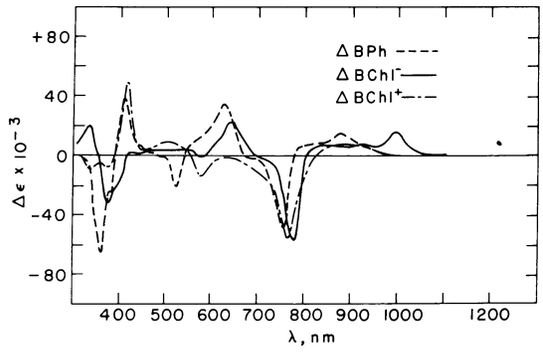

FIG. 4. Comparison of the difference spectra $\Delta BChl^- = BChl^- - BChl$ in dimethylformamide (—); $\Delta BChl^+ = BChl^+ - BChl$ (— · —) and $\Delta BPh^- = BPh^- - BPh$ (- - - -) in CH_2Cl_2.

−0.55 V for the reduction of BPh, E_m = +0.45 V for the oxidation of P_{870}, and therefore $\Delta E \simeq 1$ V or 23 kcal/mol[‖]. An incident photon of 870-nm wavelength represents an energy of 1.43 eV (33 kcal/mol). A potential separation of 1 V (23 kcal/mol) thus means that about 70% of the incident light energy has been converted into chemical energy. The agreement may be fortuitous, but Duysens calculated (42) on thermodynamic grounds, that in purple bacteria, the maximal efficiency is 68% for the conversion of radiant energy into free energy, for diffuse 850- to 880-nm light. Ross and Calvin similarly predicted a maximum potential difference of 0.9 eV in *Chromatium* for light intensities that approximate natural conditions (43). Other calculations on energy efficiency (44) also indicate that less than 100% of the energy incident on a photosynthetic system can be converted into chemical energy.

These calculations and the psec experimental data help to exclude the possibility that charge separation occurs within the BChl complex before the electron "tunnels" out. The optical changes that the formation of the BChl cation and anion radicals would cause seem incompatible with the observed P^F spectrum (Fig. 4): the 530-nm BPh band will not bleach and no increase in absorption will take place in the 1050- to 1300-nm region (17, 33). The kinetic sequence is clear:

$$P^*_{870} \longrightarrow BChl^+ + BChl^- \longrightarrow P^+_{870} + X^-$$

and P^+_{870} should appear as the ion pair disappears and X is reduced. This is not observed; the absorption bands above 1050 nm characteristic of P^+_{870} appear immediately upon excitation (15).

The creation of the radicals $BChl^+$ and $BChl^-$ would also result in a net energy difference of 1.5 V in CH_2Cl_2 or butyronitrile (+0.64 for oxidation and −0.86 for reduction). (We assume that $\Delta G \geq 0$ for the disruption of the dimer, $P_{870} \rightarrow$ 2 BChl, required to balance the reaction: $P_{870} \rightarrow$ 2 BChl $\rightarrow$ $BChl^+ + BChl^-$.) The value of 1.5 V, even if overestimated, implies that the incident 870-nm light (1.43 eV) is converted into chemical energy with an efficiency ≥100% for a one photon event.

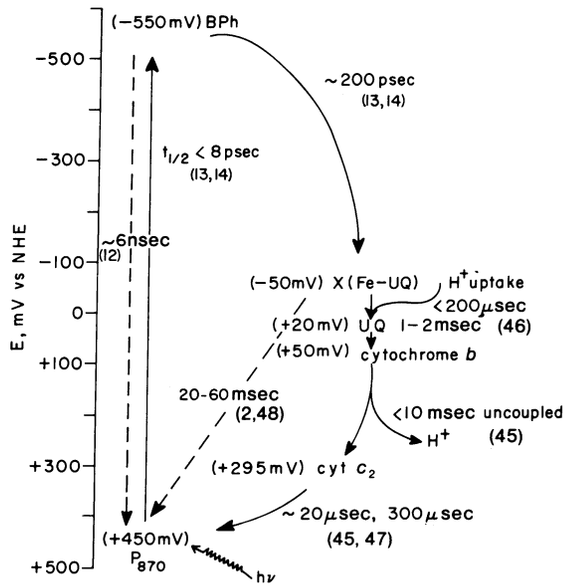

FIG. 5. Photosynthetic scheme that incorporates BPh in the cyclic electron transport of *R. spheroides*. Half-time for each reaction is indicated by the adjacent number, with references in parentheses.

We consider one additional possibility: $P_{870}^* \rightarrow P^+_{870} + BChl^-$. ΔE for this reaction is estimated at 1.3 V or 30 kcal/mol (E_m = 0.45 V for the oxidation of P_{870} and $E_{1/2}$ = −0.86 for the reduction of BChl in CH_2Cl_2).

This again seems to be too high an energy conversion (>90%) but more tolerable than the $BChl^+ + BChl^-$ reaction. The optical changes for this reaction can be predicted by comparing the difference spectra for BChl and BPh shown in Fig. 4. No changes occur at 530 nm where BPh absorbs, and a band at 1000 nm is observed in ΔBChl. For X not reduced, $P_{870}^* \rightarrow P^+_{870} + BChl^- \rightarrow P^+_{870} + BChl + X^-$, and therefore the 1000-nm band of $BChl^-$ should appear originally and then disappear while no change should take place at 530 nm. Within experimental error, the kinetic data at 530 and 1000 nm do not support the predicted sequence (15), and we conclude that within the time and spectral resolution of the present experiments, intra-bacteriochlorophyll charge separation does not occur.

If the reaction center can be poised at a potential low enough so that BPh is reduced (this can be ascertained by the appearance and/or disappearance of the BPh^- and BPh spectra), then the formation of P^F should be prevented upon excitation. The charge separation may then be localized to the bacteriochlorophylls (5) or the system may simply decay via a triplet or fluorescence.

In summary, the charge separation into oxidized BChls and reduced BPh that we have proposed seems compatible with existing optical, kinetic, thermodynamic, fluorescence, and triplet data. We have therefore incorporated BPh into the known cyclic electron transport processes (45) that occur in *R. spheroides* (Fig. 5).

We are indebted to Drs. M. D. Archer and S. W. Feldberg for stimulating discussions on electrochemistry, to Dr. R. K. Clayton for providing us with the original data of ref. 35, to Dr. A. Delgado for cyclic voltammetry measurements, to the authors of refs. 12–15, particularly Drs. P. L. Dutton, K. J. Kaufmann, and W. W. Parson, for valuable discussions and for keeping us informed of their results,

[‖] Junction potentials are inherent in the $E_{1/2}$ measurements because of the calomel electrode used as reference. To minimize the effect, we averaged $E_{1/2}$ values obtained in four different solvents. Junction potentials and other solvation effects can be cancelled by using the oxidation potential of BChl in CH_2Cl_2, $E_{1/2}$ = +0.64 V against NHE. ΔE for $BChl^+ + BPh^-$ is then equal to 1.2 V. In chromatophores, P_{870} undergoes oxidation before the antenna BChl (41), and the oxidation potential of P_{870} is therefore less than that of BChl. The upper limit of ΔE for the creation of P^+_{870} and BPh^- will hence be less than 1.2 V.

and to Drs. M. C. Thurnauer, J. R. Norris, J. J. Katz, R. E. Connors, and R. H. Clarke for preprints of their triplet work (38, 40). This work was performed under the auspices of the U.S. Energy Research and Development Administration.

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