MULTI-CHANNEL FLASH SPECTROSCOPY OF THE REACTION CENTERS OF WILD TYPE AND MUTANT *Rhodobacter sphaeroides*: BACTERIOCHLOROPHYLL*α*-MEDIATED INTERACTION BETWEEN THE CAROTENOID TRIPLET AND THE SPECIAL PAIR†

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Abbreviations: B*α*,*β*, accessory bacteriochlorophylls on *A*- and *B*-branches; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Car, carotenoid; D*α*,*β*, bacteriochlorophylls of the special pair on *A*- and *B*-branches; H*α*,*β*, bacteriopheophytins on *A*- and *B*-branches; LDAO, lauryldimethylamine oxide; NaAsc, sodium ascorbate; NaDT, sodium dithionite; O₂(Δ₂), singlet delta oxygen; P₈₇₀, special pair; Qₐ, primary quinone acceptor; Q₉, secondary quinone acceptor; *Rb*, *Rhodobacter*; RC, reaction center; *Rsp.*, *Rhodospirillum*; TmS, time-resolved triplet-minus-singlet; ΔA(λ,t), time-resolved flash-induced changes in absorbance

† This paper is dedicated to the memory of Prof. Arnold J. Hoff.
ABSTRACT

Multi-channel flash spectroscopy (with microsecond time resolution) has been applied to carotenoid (Car) containing and carotenoidless reaction centers (RC's) of *Rhodobacter sphaeroides*, with a view to investigating the interaction between the Car and its neighbouring pigments at room temperature. Under neutral redox potential conditions, where the primary quinone acceptor ($Q_A$) is oxidized, the light-induced spectral changes in the 350–1000 nm region are attributed to the photochemical oxidation of the special pair ($P_{870}$), the generation of $P_{870}^+Q_A^-$, and the attendant electrochromism of adjacent chromophores. A bathochromic shift of <1 nm in the visible absorption region of Car reveals the sensitivity of Car to the $P_{870}$ photooxidation. Under low redox potential conditions, where $Q_A$ is reduced, $P_{870}$ triplets ($P_{870}^\dagger$) are formed. The triplet-minus-singlet (TmS) spectrum of carotenoidless RC's shows a deep bleaching at 870 nm, which belongs to $P_{870}^\dagger$, and additional (but smaller) bleaching at 800 nm; the entire spectrum decays at the same rate (with a lifetime of about 50 μs). The bleaching at 800 nm arises from the pigment interaction between $P_{870}^\dagger$ and the accessory bacteriochlorophylls ($B_{a,b}$). In Car containing RC's, the TmS spectra of Car are accompanied by two smaller, negative signals—a sharp peak at 809±2 nm and a broad band at 870 nm—which decay at the same rate as the TmS spectrum of Car (ca. 10 μs). The former is ascribed to the perturbation, by Car$^\dagger$, of the absorption spectrum of B$_b$; the latter, to the TmS spectrum of $P_{870}^\dagger$, a species that appears to be in approximate thermal equilibrium with Car$^\dagger$. These assignments are consistent with the absorption-detected magnetic resonance spectra obtained by other workers at low temperatures.
INTRODUCTION

Carotenoid (Car) pigments are known to play several roles in photosynthesis (1). First, they confer photoprotection to chlorophyll (Chl) and bacteriochlorophyll (BChl) pigments through a twofold strategy: preventing the formation of singlet oxygen, $O_2(^1\Delta_g)$, by quenching a potential donor triplet, and quenching $O_2(^1\Delta_g)$ that does get formed as a result of energy transfer from a triplet (B)Chl. Secondly, Car's protect the photosynthetic apparatus of green plants by dissipating excess energy through a process, known as nonphotochemical quenching, whose details are still a matter of much debate (2-7). Thirdly, Car's function as light harvesting pigments by transferring energy from their singlet excited states to lower lying singlet states of (B)Chl (8-10). Finally, they can also serve as structural elements, vital for maintaining apoproteins and (B)Chl's assembled in the photosynthetic complexes (11,12). It is generally agreed that photoprotection, recognized first by Griffiths and co-authors (13), is the most important of the functions mentioned above.

The physical basis of photoprotection is triplet-triplet energy transfer, a process that requires electron exchange (14). A Chl-like molecule which is in the lowest triplet state can transfer its electronic excitation to a Car not only when the electron clouds of the two molecules overlap directly but also when there is an appropriate bridge or a conduit between the two moieties. The bridge may be a third chromophore, as is the case in some reaction centers (15,16) or a so-called spacer (or linker) in a model compound (17). Intimate Car-(B)Chl contacts are common in photosynthetic systems; such contacts, and the resulting intermolecular interactions, determine the transition probabilities of various nonradiative and radiative processes of interest (transfer of an electron or energy from one chromophore to another in an assembly of contiguous chromophores and the absorption spectrum of the assembly).

When pigment-pigment interactions are weak, the observed absorption spectrum, $A(\lambda)$, of a multi-chromophore assembly can be modelled, to a rough approximation, as a superposition of
contributions attributable to the individual components (18-20). However, the inadequacy of this independent-chromophore approximation becomes apparent when one purposely perturbs—by photoexcitation, genetic manipulation, chemical treatment, or some other means—molecules of a single pigment and records \( \tilde{A}(\lambda) \), the absorption spectrum of the perturbed assembly. An examination of the difference spectrum \( \Delta A = \tilde{A}(\lambda) - A(\lambda) \) invariably reveals two types of signals: those, to be called primary absorbance changes, which clearly pertain to the perturbed pigment, and others, to be called secondary absorbance changes, which become intelligible only if one goes beyond the independent-chromophore approximation, and allows for the possibility that the perturbation, though directed primarily at molecules of one type, must affect, indirectly and to a smaller degree, unexcited molecules of other types, if they happen to be in the vicinity of the purposely perturbed molecules (21-27). A particular feature in a difference spectrum must decay, if it is of secondary origin (in the sense defined here), at the same rate as the perturbing species; however, the converse need not be true, since signals arising from distinct species which are in approximate thermal equilibrium (due to rapid interconversion) will also exhibit parallel decay (see below). If an instrument with a sufficiently wide spectral range is available, time-resolved, multi-channel flash spectroscopy is ideally suited for exploring such kinetic behaviour (24,28); by examining two spectra recorded at different times after photoexcitation, one can easily see which features in the spectrum decay at the same rate.

This work is concerned with the interactions among neighbouring pigments in the reaction center (RC) of three strains of *Rhodobacter (Rb.) sphaeroides*, namely those isolated from the strains 2.4.1 (wild type), RCO2 (antenna deficient) and R26.1 (carotenoidless). To explain the motivation for our investigation, we recall briefly the cofactors of the RC of purple bacteria, and the paths of electron transfer and triplet energy transfer. Figure 1 shows the RC cofactors in the L and M polypeptides of *Rb. sphaeroides* strain 2.4.1 (29). Each LM heterodimer houses four BChl \( a \), two bacteriopheophytin (BPh) \( a \) molecules, one Car (here 15,15’cis-spheroidene), two quinones (Q, here...
ubiquinones) and one nonheme iron atom lying between the two Q's; we note in passing that the Car in strain RCO2 is spheroidenone. The L and M polypeptides with their cofactors show a nearly 2-fold symmetry around an axis perpendicular to the plane of the intracytoplasmic membrane. The two BChl $\alpha$ molecules located at the interface of the L and M polypeptides near the periplasmic side constitute the so-called special pair, here denoted P$_{870}$. The two BChl molecules of P$_{870}$ are also called D$_A$ and D$_B$, where the subscripts $A$ and $B$ signify association with the L-dominated branch and the M-dominated branch respectively; these subscripts are also appended to the corresponding cofactors. Next to P$_{870}$ one finds the monomeric BChl's B$_A$ and B$_B$, followed by the BPh's H$_A$ and H$_B$, and finally the quinones Q$_A$ or Q$_B$. The single Car molecule bound to the M polypeptide is the most conspicuous, but not the only, symmetry-breaking element in the system. Both $A$ and $B$ branches are active, but perform different roles. Upon the absorption of a photon by P$_{870}$, an electron transfer takes place along the $A$-branch; accordingly, this is called the electron transfer active branch of RC's. Charge separation starts with the reduction, with a few ps, of H$_A$ through an electron transfer reaction via B$_A$ (30). Subsequently H$_A$ reduces Q$_A$ within about 200 ps, which in turn reduces Q$_B$ in about 25 $\mu$s (31 and references therein). When P$_{870}^+$ is not reduced by a cytochrome, the electron returns to P$_{870}^+$ by charge recombination from Q$^-_B$ (or Q$^-_A$ if the pocket of the exchangable Q$_B$ is empty or the Q$_A$-to-Q$_B$ electron transfer is inhibited). The lifetimes for the two charge recombination reactions are respectively about 1 s and 90 ms (32). When the electron transport is inhibited beyond H$_A$, because Q$_A$ is either reduced or absent in RC's, the $B$-branch, the so-called photoprotection active branch, takes over. In this case, the radical pair P$_{870}^+\Phi^+_A$ decays with a lifetime of 10-20 ns at room temperature (33), undergoing intersystem crossing, $^3[P_{870}^+H_A^+] \rightarrow ^3[P_{870}^+H_A^+]$, or internal conversion, $^1[P_{870}^+H_A^+] \rightarrow P_{870}H_A^-$. The first process is followed by $^3[P_{870}^+H_A^+] \rightarrow P_{870}^\dagger H_A^-$. However, the P$_{870}^\dagger$ so produced would not be able to transfer its triplet excitation to Car, which is situated about 1 nm away, in the absence of an intermediary, namely B$_B$;
electronic excitation is transferred from $P_{870}^\dagger$ to Car in a two-step reaction (15,16). The first step, $P_{870}^\dagger B_p Car \rightarrow P_{870}^\dagger B_p Car^\dagger$, requires thermal activation and is rate limiting; the second, $P_{870}^\dagger B_p Car \rightarrow P_{870}^\dagger B_p Car^\dagger$, is essentially activationless when $n \geq 9$, where $n$ is the number of conjugated double bonds in the Car (15,34-38). The activation energy for $P_{870}^\dagger B_p Car \rightarrow P_{870}^\dagger B_p Car$ depends on the pigment occupying the $B_p$ pocket (15,38) and also on the protein environment surrounding it (39). At room temperature the overall triplet reaction $P_{870}^\dagger B_p Car \rightarrow P_{870}^\dagger B_p Car^\dagger$ takes place in $\sim 30$ ns and $Car^\dagger$ decays in 5-7 s (34,37), but at low temperatures, $P_{870}^\dagger \rightarrow Car^\dagger$ transfer slows down considerably (16,40). The triplet excited state of $B_p^\dagger$ is believed to be rather short-lived, with a lifetime of the order of $\sim 10^{-9}$ s (16,41), which means that its concentration does not build up significantly in Car containing RC's.

Given the proximity of the three pigments (Car, $B_p$, and $P_{870}$), one would expect to see, in view of what has been said above concerning pigment-pigment interactions, both primary and secondary absorbance changes after the generation of $Car^\dagger$ in the RC's of purple bacteria. If triplet excitation resides exclusively on $Car^\dagger$, the primary changes would occur in the region (400-600 nm) where the triplet-minus-singlet (TmS) spectrum of spheroidene and spheroidenone occur (16,34); the secondary spectral changes, around 800 nm (where $B_p$ absorbs), and possibly around 870 nm. Using absorption-detected magnetic resonance (ADMR), experiments have been conducted (38,41) in which the native BCHl's of the RC of $Rb. sphaeroides$ were selectively exchanged with a related pigment whose $Q_y$ band is blue-shifted with respect to that of the native pigment. These studies, carried out at low temperatures (8–130 K), confirmed the existence of $Car^\dagger – B_p$ and $P_{870}^\dagger – B_p$ interactions. However, the authors of refs. (38) and (41) did not address the question of whether the formation of $Car^\dagger$ leads to secondary spectral changes around 870 nm. We are aware of only one publication (42) where the photo-induced difference spectra of carotenoid containing
RC's of *Rb. sphaeroides* 2.4.1 and *Rhodospirillum (Rsp.) rubrum* S1 have been recorded (under steady illumination) over the entire spectral region of interest (350–950 nm) and over a wide temperature range. However, an examination of these spectra shows that (at a temperature close to 290 K) the negative peaks in the P₈₇₀ region are of nearly the same size as the positive peaks in the region of Car triplet-triplet absorption; in other words, the supposedly secondary signal is comparable to its primary counterpart. Though at least one example is known where the primary and secondary features have comparable amplitudes (22), such behaviour is an exception rather than the rule. Since the separation between Car and P₈₇₀ is rather large, one would expect only a modest interaction between the two chromophores. The unexpectedly large size of the secondary signal in the spectra published by Lous and Hoff (42) prompted us to take a second look at the TmS spectra of the RC's of *Rb. sphaeroides*, using a multi-channel flash spectrometer with microsecond time resolution and a wide spectral span. The results of our study, which are presented here, have revealed two bleaching signals in the near infrared region, both appreciably smaller than the primary signal; one (peaking at about 807 nm) is in agreement with the ADMR results (38,41), but the other (peaking at about 870 nm) does not accord with the spectra presented in ref. (42). A discussion of this discrepancy is deferred till we reach a detailed analysis of our own results and those published by other workers.

**MATERIAL AND METHODS**

*Cell culture growth.* Cells of *Rb. sphaeroides* strains 2.4.1 and R26.1 were grown under anaerobic/light conditions at room temperature as described in (43), whereas cells of the antenna-deficient strain RCO2 of *Rb. sphaeroides* were grown under semiaerobic/dark conditions at 34º C in M22+ medium as described in (44).

*Purification of RC's.* Cells were harvested after 24-72 h growth under the above conditions. The intracytoplasmic membranes were isolated by passing the harvested cells through a French
press, followed by a centrifugation at 10 000 g for 10 min at 4°C to remove unbroken cells and an ultracentrifugation at 250 000 g for 1 h at 4°C. The intracytoplasmic membrane pellet was suspended to a concentration of about 25 absorbance units cm⁻¹ at 800 nm in 20 mM Tris-HCl pH 8.0. RC's were solubilised from the intracytoplasmic membrane suspension by the addition of the detergent lauryldimethylamine oxide (LDAO) and NaCl, the latter to a final concentration of 150 mM. A final detergent concentration of 0.25% (vol/vol) was used when isolating RC's of *Rb. sphaeroides* strains 2.4.1 and R26.1 or 1% (vol/vol) when isolating the ones from the antenna-deficient strain RCO2. The intracytoplasmic membrane suspensions were incubated in LDAO for 1.5 h at 30°C. The suspensions were then centrifuged at 250 000 g for 1 h. The supernatants containing the solubilized RC's were collected and the pellets were once again resuspended in 20 mM Tris-HCl pH 8.0. The LDAO/NaCl treatment was repeated at least two more times to achieve maximal extraction of RC's. The latter two steps were not required when extracting RC's of the antenna-deficient strain RCO2 of *Rb. sphaeroides* since the 1% (vol/vol) LDAO solubilization was enough to reach the maximal yield. Supernatants containing the solubilized RC's were diluted 2-3 fold in 20 mM Tris-HCl pH 8.0 and loaded onto a Whatman DE52 anion exchange column (Bodman Industries, Aston PA) equilibrated with 0.1% (vol/vol) LDAO, 20 mM Tris-HCl pH 8.0. A stepped gradient of increasing NaCl concentrations was used to remove free pigments, cytochromes and/or light harvesting complexes. RC fractions, eluting between 150-200 mM NaCl, were diluted 2-3 fold and were futher purified on a HiTrap Q anion exchange column (Amersham Pharmacia Biotech., Buckinghamshire England). RC's were eluted this time with a linear gradient of increasing NaCl concentration from 0-0.5 M. RC fractions with an absorbance ratio $A_{280}/A_{800}$ of ~1.3 were pooled and kept at -20°C until use.

*Transient Absorption spectroscopy.* Flash-induced changes in the absorbance of RC from *Rb. sphaeroides* were recorded with the aid of a home-built multi-channel kinetic spectrometer whose components and operation have been described in a recent publication (45). Briefly, a linear flash lamp with a pulse width of about 5 μs serves as the pump source for a sample placed in a
standard fluorescence cuvette (1 cm × 1 cm cross section). The flash-induced change in the absorbance of the sample is monitored in a right-angle geometry by using a miniature flash lamp with a pulse width of about 2 μs. The spectral analysis of the transmitted beam is carried out with the aid of bifurcated fibre optic bundle and two spectrographs, one of which covers the 200-620 nm range and the other 600-1010 range. The light dispersed by the grating in each spectrograph is detected by an ungated 512-element diode array; time resolution is achieved by varying the delay (denoted by $t$) between the firing of the pump flash and the triggering of the probe source; the overall time resolution, determined mainly by the width of the pump source, is close to 5 μs. The flash-induced changes in the absorbance of the sample are displayed in the form of a difference spectrum equalling, 

$$\Delta A(\lambda; t) = \tilde{A}(\lambda; t) - A(\lambda),$$

where $A(\lambda)$ is the absorbance of the sample before, and $\tilde{A}(\lambda; t)$ the absorbance at time $t$ after, the instant at which the pump source is fired.

**Chemical treatments.** RC's were chemically closed (or $Q_A$ was reduced) by adding sodium dithionite (NaDT) to a final concentration of 15 mM. A concentration of 5 mM atrazine was used to inhibit the $Q_A$-to-$Q_B$ electron transport. All the assay buffers were 50 mM Tris-HCl pH 8.0.

**Absorption spectrosopy.** Absorption spectra of RC's were recorded by using a commercial Shimadzu spectrophotometer Model UV-160A (Shimadzu Scientific Instruments, Inc. Columbia MD). The absorbance readings were taken at 1 nm intervals.

### RESULTS AND DISCUSSION

The results to be presented in this section fall into three groups: the absorption spectra of the RC's of the three strains, difference spectra representing flash-induced transient absorbance changes in the RC's under “neutral” redox conditions, and similar spectra under low redox potential. The absorption spectra of the RC's containing spheroidene or spheroidenone or no caroteoids are already known (16,18), and they are presented here only for the readers’ convenience. Photo-induced
difference spectra of the RC of carotenoid deficient *Rb. sphaeroides* under neutral conditions and under low redox potential are also available in the literature; our spectra are presented here partly because we have covered a wider spectral range, which has allowed us to infer the charge on Qₐ in the latter case, but mainly because they will prove to be relevant when we come to compare our results with those in ref. (42). The new results in the present paper pertain to RC's of carotenoid containing strains; previous flash-induced difference spectra (16,34) of these systems leave unanswered the principal issue examined here, for they cover only the region where the difference spectrum is dominated by the TmS spectrum of Car: 400-600 nm for spheroidene and 400-650 for spheroidenone.

**Ground state absorption spectra of RC's**

The absorption spectra of RC's from the wild type, carotenoidless, and antenna-deficient strains 2.4.1, R26.1 and RCO2 of *Rb. sphaeroides* are shown in Fig. 2; these plots are in complete agreement with earlier reports (44,46). A cursory examination of the three spectra is sufficient to show that the most conspicuous differences are found in the region where Car pigments absorb. As expected on the basis of the published spectra (47), the absorption spectrum of spheroidene, which constitutes about 90% of the Car content in the wild type strain 2.4.1, shows considerable structure, whereas the spectrum of spheroidenone, the major carotenoid in the antenna-deficient strain RCO2 (44), is essentially structureless (Fig. 2 inset).

**Flash-induced difference spectra of RC's under neutral redox potentials**

The flash-induced absorbance changes in the RC's of *Rb. sphaeroides* strains 2.4.1 and R26.1 are shown in Fig. 3. The two difference spectra have been normalized to \( \Delta \varepsilon_{870} = -112 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \),
which is the expected differential molar absorption coefficient (Δε) for the absorbance change at 870 nm for complete photooxidation of 1 M P_{870} (48). One sees a prominent bleaching in the near infrared region due to the photooxidation of P_{870} (49). On our time scale, photooxidation results in the reaction P_{870}^+ Q_A^- . In the absence of interactions, one would only expect to see, in a difference spectrum, negative signals due to the loss of P_{870} and Q_A, and positive signals due to the appearance of P_{870}^+ and Q_A^- . Part of the 600 nm negative signal and of the 425 nm and 545 nm positive signals in Fig. 3 are due to the loss of P_{870} and gain of P_{870}^+, respectively (18). The negative signal peaking at 272 nm has contributions from P_{870} and Q_A. Using the published values for the molar absorption coefficients of the ubiquinone and the anionic semiubiquinone in methanol,

$$\Delta \varepsilon_{274} = 15.1 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \text{ and } \Delta \varepsilon_{274} = 2.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1},$$

respectively (50) and the Δε of the oxidized minus reduced spectrum of BChl in CH_{3}OH, $\Delta \varepsilon_{272} = -6.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (51), and assuming that these values are applicable to Q_A and P_{870}, one gets $\Delta \varepsilon_{272-274} = -19.3 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. On comparing this theoretical overall Δε value with the one experimentally obtained for 1 M RC of *Rb. sphaeroides* 2.4.1 and R26.1 the data shown in Fig. 3 are in a good agreement with this prediction. This suggests that the electron charge is stabilized in Q_A. Indeed, the addition of 5 mM atrazine also confirmed it, since no change in ΔA could be detected in the 450 nm region (data not shown), where Q_A^- or Q_B^- is most conveniently measured, and the subsequent formation of Q_A^-Q_B^- leads to a decrease in ΔA (52). Since the time resolution in our instrument is about 5 μs, no trace of the anionic $\Phi_A^-$, with an expected lifetime of about 250 ps (53), is observed in the difference spectra shown in Fig. 3.

In the near infrared region, a hypsochromic shift of the absorption band of the B_{A,B} and a bathochromic shift of the absorption band of H_{A,B}, partly obscured by the larger hypsochromic shift of B_{A,B}, are linked to the photooxidation of P_{870}. Similar but smaller changes in the Soret band are also present. Together with B_{A,B} and H_{A,B}, Car's are also sensitive to changes in the internal electrical
field upon photooxidation of $P_{870}$ (54,55). In the visible absorption region of Car, the flash-induced difference spectrum of RC’s of *Rb. sphaeroides* 2.4.1 shows features that suggest a bathochromic shift in the ground state absorption of spheroidene (Fig. 3 inset). Simulations, where the ground state absorption of spheroidene is red shifted, with the aim of matching the flash-induced changes in the 430-530 nm region, indicate that the shift is <1 nm in RC’s.

**Flash-induced difference spectra of RC’s under low redox potentials**

We begin by discussing the spectra arising from the formation of $P_{870}^\dagger$. Time-resolved TmS spectra of RC's of *Rb. sphaeroides* strain R26.1 in the presence of 15 mM NaDT are shown in Fig. 4. In the blue-green absorption region, the difference spectrum shows a bleaching of the absorption bands near 600 nm and below 400 nm, and also the appearance of new (and broad) absorption bands centered at ~430 and ~510 nm. All these features have their counterparts in the TmS spectrum of BChl in organic solvents (56,57). In the near infrared region, the TmS spectrum of the RC shows a deep bleaching that is due to the formation of $P_{870}^\dagger$. The lifetime for $P_{870}^\dagger$ estimated from the spectra in Fig. 4 comes out to be about 50 µs. Previous works have shown that the lifetime depends on the temperature and the charge on Q$_A$ (58,59); the half life at room temperature has been reported to be 12 µs in the presence of Q$_A$ and 90 µs if Q$_A$ is fully reduced or altogether removed; other workers, using 5 mM sodium ascorbate (NaAsc), have reported values of about 60 and 26 µs for the lifetime at room temperature (37,60).

A closer inspection of the difference spectrum in Fig. 4 reveals at least one feature which is absent in the TmS spectrum of BChl in organic solvents: a bleaching signal at 800 nm. This bleaching differs, in shape as well as in rate of decay, from that observed at 800 nm upon illumination of RC’s of *Rb. sphaeroides* under neutral conditions (Fig. 3) and found to persist for several milliseconds. The 800 nm bleaching in Fig. 4 decays with nearly the same lifetime as $P_{870}^\dagger$;
it has also been observed previously (18,57) and assigned to the $B_{A,B}$ band of RC's. Microwave induced absorption (MIA) spectra of RC's of *Rb. sphaeroides* at 8 K have suggested that there exists a (weak) interaction between $P_{870}^\dagger$ and $B_{A,B}$ (38). Although our TmS spectra have been recorded at room temperature and a direct comparison cannot be made with the former MIA spectra, the similarity in the decay rates of $P_{870}^\dagger$ and the 800 nm bleaching in RC's of *Rb. sphaeroides* R26.1 suggests that the 800 nm bleaching is a secondary feature, thereby so supporting the view of Hartwich *et al.* (38).

We have now set the stage for discussing the flash-induced changes under conditions where the primary quinone acceptor $Q_A$ is kept reduced with 15 mM NaDT, and a higher $P_{870}^\dagger$ yield is obtained because the triplet $P_{870}^\ddagger$ is produced *via* the radical pair mechanism,

\[
\frac{1}{2}[P_{870}^+ H^-_A] \rightarrow \frac{3}{2}[P_{870}^+ H^-_A] \rightarrow P_{870}^\ddagger H^-_A \quad (61).
\]

Triplet energy is then transferred to Car from $P_{870}^\dagger$. Figure 5a shows the flash-induced difference spectra of RC's of *Rb. sphaeroides* strain 2.4.1 in the presence of 15 mM NaDT at two delays (2 and 11 $\mu$s); Fig. 5b gives similar plots for the antenna-deficient strain RCO2. To demonstrate that the entire spectrum decays at the same rate, the $\Delta A(\lambda; 11 \mu s)$ in each case has been multiplied by a numerical factor $N$ to match $\Delta A(\lambda; 2 \mu s)$. The spectra described by Schenck *et al.* (16) and Cogdell *et al.* (34) covering the 400–600 nm region agree extremely well with those plotted in Figs. 5a and 5b, respectively; this part of the spectrum can therefore be assigned, following these authors, to the TmS spectrum of spheroidene and spheridenone in the RC of *Rb. sphaeroides* strain 2.4.1 and strain RCO2, respectively. It is pertinent to add that the Car in the RC of *Rsp. rubrum* S1 is spirilloxanthin (34), and that the room temperature TmS spectrum of this RC is almost indistinguishable from that of *Rb. sphaeroides* RCO2 (shown in Fig. 5b). This similarity is due to the fact that both spirilloxanthin and spheridenone are more fully conjugated variants of spheroidene; for the purpose of this discussion, we will treat spirilloxanthin and spheridenone as equivalent. The lifetime of Car$^\dagger$ in RC's of *Rb.*
strain 2.4.1 and RCO2, as judged from the time-resolved spectra, is \( \text{ca. 10 \mu s} \), close to the value reported in ref. (34).

When one looks at the infrared part of the spectra in Fig. 5, one sees that the flash-induced absorbance changes in the RC’s of the two strains 2.4.1 and RCO2 of \textit{Rb. sphaeroides} contain two negative signals at 809±2 and 870 nm, here to be denoted as \( N_{800} \) and \( N_{870} \), respectively; these wavelengths correspond, it should be recalled, to the peak absorbances of \( B_{4,8} \) and \( P_{870} \) pigments. Clearly, these negative peaks cannot be interpreted as a part of the TmS spectrum of Car, since the ground state of spheroiden(on)e does not absorb in the \( Q_y \) region of \( B_{4,8} \) and \( P_{870} \). Within the uncertainty arising from the noise, both \( N_{800} \) and \( N_{870} \) seem to decay at the same rate as the Car TmS signal. The claim that one or both of these negative peaks arise from an interaction of the relevant pigment with \( \text{Car}^\dagger \) would be consistent with the fact that the entire spectrum in each panel of Fig. 5 decays at the same rate. However, an alternative, which is equally consistent with the observations must now be mentioned: parallel decay of two signals can be a consequence of an approximate thermal equilibrium between the corresponding species. We pause therefore to say a few words about approximate equilibrium.

Let us consider the following reaction scheme,

\[
\begin{align*}
\text{D} & \overset{k_4}{\rightleftharpoons} 3\text{D} \overset{k_1}{\longrightarrow} 3\text{C} \overset{k_3}{\longrightarrow} \text{C},
\end{align*}
\]

(Scheme I)

where \( 3\text{D} \) and \( 3\text{C} \) stand for \( P_{870}^\dagger \) and \( \text{Car}^\dagger \), respectively, and \( \text{D} \) and \( \text{C} \) stand for \( P_{870} \) and \( \text{Car} \), respectively. The notation (for the species and the rate constants) has been chosen so as to facilitate comparison with a scheme analysed by Lous and Hoff (42), who set \( k_4 = 0 \). Though only two pigments are explicitly indicated, the scheme can be applied to a wild type RC, since triplet excitation flows out from the intermediary pigment at a very rapid rate; indeed, the flow of triplet excitation from \( P_{870}^\dagger \) to \( \text{Car}^\dagger \) would be too slow, in the absence of the intermediary pigment, to allow the establishment of approximate thermal equilibrium between the two species. One can easily
verify that, if \( k_m \ll k_1 + k_2 \), where \( m = 3 \) or \( 4 \), \(^3\)D and \(^3\)C will decay at the same rate such that their ratio \( \frac{^3D}{^3C} = \frac{k_2}{k_1} \) remains constant. We will refer to this state of affairs as *approximate thermal equilibrium*, reserving the term equilibrium for the situation when \( k_m = 0 \). It is evident that the foregoing discussion can be applied to a carotenoidless RC simply be replacing Car with \( B_B \).

It follows from the above that either of the two negative peaks (N\(_{800}\) and N\(_{870}\)) might be a secondary feature, signifying an interaction between the corresponding pigment (\( B_B \) and P\(_{870}\), respectively) and Car\(^\dagger\), or a primary feature, signifying approximate thermal equilibrium between Car\(^\dagger\) and the triplet of the corresponding pigment (\( B_B \) and P\(_{870}\), respectively). Previous investigators (38,41,42) have concluded that N\(_{800}\) (or its equivalent) can be attributed to an interaction between Car and \( B_B \); it should be noted that the negative peak can be seen even at 8 K (38,41), which rules out the possibility that it represents a species in approximate thermal equilibrium with Car\(^\dagger\).

Accordingly, we interpret N\(_{800}\) in the transient spectra of RC’s of *Rb. sphaeroides* strains 2.4.1 and RCO2 as a perturbation of the ground state of \( B_B \) by the formation of Car\(^\dagger\); the perturbation, persists only as long as the Car\(^\dagger\) population itself, which explains why N\(_{800}\) decays at the same rate as the Car TmS signal.

Turning now to N\(_{870}\), we recall the difference spectra of *Rb. sphaeroides* 2.4.1 and *Rsp. rubrum* S1 (comparable, in the light of what has been said above, to *Rb. sphaeroides* RCO2) published by Lous and Hoff (42). Their spectra, recorded at several temperatures between 10 K and 290 K, cover the spectral range 350-950 nm; in the 400-780 nm region, their 288 K spectrum (with a resolution of 3.2 nm) of *Rb. sphaeroides* is in very good agreement with the spectrum in Fig. 5a, and their 287 K spectrum of *Rsp. rubrum* bears great similarity to the spectrum in Fig. 5b. However, we encounter serious disagreements when we move to longer wavelengths.

To discuss the source of the above mentioned discrepancy between our spectra and those of Lous and Hoff (42), we recall first that their spectra were recorded in the presence of 10 mM NaAsc
and ours in the presence of 15 mM NaDT. We have found that 10 mM NaAsc is insufficient for complete reduction of $Q_a$ in our preparations; assuming, for the sake of argument, that Lous and Hoff (42) also faced (but did not recognize) this difficulty, one is led to conclude that a difference spectrum recorded under their experimental conditions will be a mixture of the spectra appearing in Figs. 3 and 5a. To simulate these conditions, we synthesized various linear combinations of the two spectra, but the resulting curves did not agree satisfactorily with the spectra in ref. (42). We were therefore led to consider a second possibility, which has been raised more than once (16,41).

Schenck et al. (16) noted that a small amount of $P_{870}^\dagger$ formed in their samples at room temperature, and suspected that this could be due to “an artefact of the reaction center preparation”. They commented that their detergent solubilization procedure could have interfered with triplet energy transfer in a small fraction of RC's, or, alternatively, “a small fraction of the reaction centers (≈10%) could simply be missing a carotenoid”. Angerhofer et al. (41) were led to advance a similar proposal because they found a lifetime of about 100 µs for $Rb. sphaeroides$ strain 2.4.1 at temperatures between 30 and 160 K, close to the decay rate in the carotenoidless $Rb. sphaeroides$ strain R26.1. If we allow for the possibility that the preparations examined in ref. (42) were defective in the sense that they contained a small fraction where the Car pigment had been dislodged and triplet transfer blocked, it follows that their difference spectra (at a temperature close to 290 K) must be superpositions of the TmS spectra of wild type and carotenoidless RC's. With this assumption, we were able to simulate spectra that seemed to be in excellent visual agreement with the spectra of Lous and Hoff (42); that the signals from the minority species ($P_{870}^\dagger$) and the majority species (Car$^\dagger$) were comparable (at about 290 K) can be easily understood when one takes account of the fact that these authors recorded their spectra under photostationary conditions, where the standing concentration of a species would be inversely proportional to its lifetime. If one considers a situation where 10% of the RC's are defective, and takes the lifetimes of Car$^\dagger$ and $P_{870}^\dagger$ to be 5 and 50 µs, respectively, one sees that the relative populations come out to be 0.9 and 1, respectively.
In our study the decay time for $N_{870}$ in the carotenoid containing RC's of *Rb. sphaeroides* is ca. 10 µs, considerably shorter than the value (ca. 50 µs) found for the decay time for $P_{870}^{\dagger}$ in the carotenoidless *Rb. sphaeroides* R26.1 (Fig. 4). Had our preparations of the RC’s of strain 2.4.1 contained a small fraction of defective RC’s, the contamination would have led to a spectrum with a time-dependent shape; at times longer than 30 µs, the Car$^{\dagger}$ population would have fallen to a negligible level, and the residual signal would have resembled the TmS signal from $P_{870}^{\dagger}$. The observed equality of the lifetime of $N_{870}$ and the rest of the difference spectrum can be attributed either to an interaction between Car$^{\dagger}$ and $P_{870}$, or to the establishment of an approximate thermal equilibrium between $P_{870}^{\dagger}$ and Car$^{\dagger}$ (see Scheme I). Since the ADMR spectra at low temperatures (41) do not show an equivalent of $N_{870}$, the first alternative can be discarded. Some additional support for favouring the second assignment comes from the fact that the relative height of $N_{870}$ in Fig. 5b is lower than that in Fig. 5a; since, the triplet energy of spheroidenone is believed to be lower than that of spheroidenone (16), it follows that, if other things are equal, the relative population of $P_{870}^{\dagger}$ in RC’s of strain RCO2 will be lower than that in strain 2.4.1.

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REFERENCES


CAPTIONS FOR FIGURES

Figure 1. Three dimensional arrangement of the cofactors housed in the L and M polypeptides of the reaction center of *Rhodobacter sphaeroides* 2.4.1. The phytyl and isoprenoid side chains of the cofactors are omitted for the sake of clarity.

Figure 2. Absorption spectra of reaction centers of the wild type (top line, $A_{\text{wt}}$), antenna-deficient (middle line, $A_{\text{ad}}$) and carotenoidless (bottom line, $A_{\text{cd}}$) strains 2.4.1, RCO2 and R26.1 of *Rhodobacter sphaeroides*. Inset: Difference absorption spectra $A_{\text{wt}} - A_{\text{cd}}$ (solid line) and $A_{\text{ad}} - A_{\text{cd}}$ (gray line).

Figure 3. Flash-induced difference spectra of the reaction centers of the wild type strain 2.4.1 (solid line) and carotenoidless strain R26.1 (gray line) of *Rhodobacter sphaeroides* under "neutral" redox potential. Inset shows a close-up of the wavelength region where spheroidene absorbs strongly. The $y$-axis was scaled by using the delta molar extinction coefficient of photochemical reaction centers of *Rhodobacter sphaeroides* determined by (48). Each spectrum is an average of 200 traces. The delay is 5 $\mu$s.

Figure 4. Triplet-minus-singlet spectra of the reaction center at several delays of the carotenoidless strain R26.1 of *Rhodobacter sphaeroides* under low redox potential (15 mM sodium dithionite). Each spectrum is the average of 256 traces.

Fig. 5. Triplet-minus-singlet spectra of the reaction centers of the wild type strain 2.4.1 (panel a) and the antenna-deficient strain RCO2 (panel b) of *Rhodobacter sphaeroides* at two different delay times (2 $\mu$s, solid line and 11 $\mu$s, open circle) under low redox potential (15 mM sodium dithionite).
The spectrum at the longer delay has been multiplied by a numerical factor $N$ to match the spectrum at shorter delay. Each spectrum is the average of 256 traces.
Fig. 1

Electron Transfer Active or *A*-Branch

Photoprotection Active or *B*-branch
Fig. 2
Fig. 3
Fig. 4
Fig. 5

\[ N = 2.25 \]

\[ N = 2.50 \]