Biochimica et Biophysica Acta, 462 (1977) 467–490

BBA 47389

SPECTROSCOPIC PROPERTIES OF THE INTERMEDIARY ELECTRON CARRIER IN THE REACTION CENTER OF *RHODOPSEUDOMONAS VIRIDIS*

EVIDENCE FOR ITS INTERACTION WITH THE PRIMARY ACCEPTOR

ROGER C. PRINCE, DAVID M. TIEDE, J. PHILIP THORNBER[a],* and P. LESLIE DUTTON

Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pa. 19174, and [a]Biology Department, Brookhaven National Laboratories, Upton, Long Island, N.Y. 11973 (U.S.A.)

(Received April 5th, 1977)

SUMMARY

The spectroscopic properties of the intermediary electron carrier (I), which functions between the bacteriochlorophyll dimer, $(\mathrm{BChl})_2$, and the primary acceptor quinone · iron, QFe, have been characterized in *Rhodopseudomonas viridis*. Optically the reduction of I is accompanied by a bleaching of bands at 545 and 790 nm and a broad absorbance increase around 680 nm which we attribute to the reduction of a bacteriopheophytin, together with apparent blue shifts of the bacteriochlorophyll bands at 830 and possibly at 960 nm. Low temperature electron paramagnetic resonance analysis also reveals complicated changes accompanying the reduction of I. In chromatophores $\mathrm{I}^{\cdot-}$ is revealed as a broad split signal centered close to *g* 2.003, which is consistent with $\mathrm{I}^{\cdot-}$ interacting, via exchange coupling and dipolar effects, with the primary acceptor $\mathrm{Q}^{\cdot-}\mathrm{Fe}$. This is supported by experiments with reaction centers prepared with sodium dodecyl sulfate, which lack the $\mathrm{Q}^{\cdot-}\mathrm{Fe}$ *g* 1.82 signal, and also lack the broad split $\mathrm{I}^{\cdot-}$ signal; instead, $\mathrm{I}^{\cdot-}$ is revealed as an approximately 13 gauss wide free radical centered close to *g* 2.003. Reaction centers prepared using lauryl dimethylamine *N*-oxide retain most of their $\mathrm{Q}^{\cdot-}\mathrm{Fe}$ *g* 1.82 signal, and in this case $\mathrm{I}^{\cdot-}$ occurs as a mixture of the two EPR signals described above. However, the optical changes accompanying the reduction of $\mathrm{I}^{\cdot-}$ are very similar in the two reaction center preparations, so we conclude that there is no direct correlation between the two optical and the two EPR signals of $\mathrm{I}^{\cdot-}$. Perhaps the simplest explanation of the results is that the two EPR signals reflect the reduced bacteriopheophytin either interacting, or not interacting, with $\mathrm{Q}^{\cdot-}\mathrm{Fe}$, while the optical changes reflect the reduction of bacteriopheophytin, together with secondary, perhaps electrochromic effects on the bacteriochlorophylls of the reaction center. However, we are unable to eliminate completely the possibility

---

A portion of this research was carried out at Brookhaven National Laboratory under the auspices of the Energy Research and Development Administration.

* Permanent address: Department of Biology and Molecular Biology Institute, University of California, Los Angeles, Calif. 90024, U.S.A.

that there is also some electron sharing between the reduced bacteriopheophytin and bacteriochlorophyll.

INTRODUCTION

The early light-induced events of purple bacterial photosynthesis occur within the photochemical reaction center · protein complex, which is now accepted to contain six chromophores; two molecules of bacteriopheophytin (BPh) and four of bacteriochlorophyll (BChl) [1]. Two of these bacteriochlorophylls comprise the bacteriochlorophyll dimer or "special pair" (designated $(BChl)_2$) which undergoes stable oxidation in the light to form $(BChl)_2^{+\cdot}$.

Early in 1975 Parson et al. [2] revealed an excited state of the reaction center (designed P^F) with a complicated spectrum involving bleaching of the $(BChl)_2$ at 865 and 605 nm, together with an apparent blue shift of the 800 nm absorbing bacteriochlorophylls and changes at 542 and 760 nm which could be attributed to reduction of bacteriopheophytin. The P^F state was subsequently shown to form within 10 ps of an actinic flash of light [3, 4], and, under conditions where normal forward photochemistry could occur, the bleaching at 542 nm relaxed with a halftime of 100–200 ps leaving optical changes associated with long-lived $(BChl)_2$ oxidation. The 100–200 ps decay was interpreted as the electron transfer rate to the "primary electron acceptor", which is now generally considered to be a quinone · iron complex (QFe). The finding that the prior chemical reduction of QFe [2–4] or the reversible removal of the Q [5] prevented the 100–200 ps decay supported the contention that the intermediary P^F state donated an electron directly to QFe. Subsequently it was shown that the actual photo-oxidation of $(BChl)_2$, (as measured by the appearance of an infrared absorbance band at 1250 nm which is considered unique to $(BChl)_2^{+\cdot}$) occurred within 10 ps, independent of the presence of Q [12] or the state of reduction of the QFe [6]. This revealed the existence of an intermediary electron carrier (designated I) operating between $(BChl)_2$ and QFe, which apparently underwent reduction in less than 10 ps concomitant with $(BChl)_2$ photo-oxidation, and subsequently reduced QFe with a halftime of 100–200 ps; in other words, the state P^F in fact represented $(BChl)_2^{+\cdot}I^{-\cdot}$ [6]. At the same time Fajer et al. [7] were examining the in vitro spectral properties of bacteriopheophytin and its radical anion, $BPh^{-\cdot}$, and they concluded that the intermediary state was $(BChl)_2^{+\cdot}\ BPh^{-\cdot}$ [7]. However, this was by no means certain since $I^{-\cdot}$ had only been seen on a transient basis, and then only in the presence of $(BChl)_2^{+\cdot}$. Attempts have therefore been made to obtain $(BChl)_2 \cdot I^{-\cdot}$ in a stable state, so that it could be studied by conventional analytical techniques without spectral interference from $(BChl)_2^{+\cdot}$. One approach has been the photochemical trapping of $I^{-\cdot}$; this has been achieved in a variety of bacterial species, but is done most readily in those species which possess low potential *c*-type cytochromes which are capable of rapid (in the μs time range at physiological temperatures, see ref. 8 for a recent review) irreversible electron donation to the photo-oxidized $(BChl)_2^{+\cdot}$. Thus, starting with reaction centers poised at a redox potential where all the components except I are in the reduced form (i.e., ferro *c* $[(BChl)_2I]Q^{-\cdot}Fe$), prolonged illumination results in the reduction of I solely at the expense of the oxidation of one cytochrome heme (i.e., illumination converts the reaction center to the state ferri *c* $[(BChl)_2\ I^{-\cdot}]Q^{-\cdot}Fe$, see refs. 9–13). Prolonged illumination is required because the rate of the reverse reaction, $I^{\cdot-} \rightarrow (BChl)_2^{\cdot+}$ is about two orders of magnitude faster than cytochrome $c \rightarrow (BChl)_2^{\cdot+}$, so only infrequently is I "stably" reduced. This trapped state is stable for long periods of time in the dark at low temperatures [10–13], but is readily relaxed at room temperatures under appropriate conditions of ambient redox potential [10–18].

The optical changes associated with the photoreduction of I have been studied in several bacteria, including *Chromatium vinosum* [11–14], *Chromatium minutissimum* [15], *Thiocapsa roseopersicina* [15] and *Rhodopseudomonas viridis* [13, 16–18]. In all cases the changes have suggested the involvement of more than one species; an apparent reduction of bacteriopheophytin together with an apparent shift and perhaps a bleaching of the 800 nm bacteriochlorophylls (which absorb at 830 nm in the bacteriochlorophyll *b*-containing *Rps. viridis*). Similarly, the low temperature electron paramagnetic resonance (EPR) spectra of the trapped $I^{\cdot-}$ state in *C. vinosum* have also revealed a complicated situation with two separate components; a narrow free radical signal, centered close to *g* 2.003, together with a broad split signal with peak maxima at *g* 2.034 and *g* 1.976 [10–12].

An alternative approach to obtaining I in a stably reduced state is direct chemical reduction, although this has so far only been achieved in *Rps. viridis* [19]. This species differs from most other isolated photosynthetic bacteria in that it contains a chlorophyll (bacteriochlorophyll *b*) which absorbs at slightly longer wavelengths both in vivo and in vitro than the bacteriochlorophyll *a* present in most species. In a previous paper [19] we discussed how this difference in wavelength maxima might result in a smaller redox potential difference between the $(BChl)_2/(BChl)_2^{\cdot+}$ and $I/I^{\cdot-}$ couples, and how this could result in the equilibrium E_m of the $I/I^{\cdot-}$ couple being within reasonable experimental range. By assuming that at low temperatures the light-induced spin polarized triplet of the reaction center $(BChl)_2$ [20–23] was an obligatory decay product of the photo-induced $(BChl)_2^{\cdot+}\ I^{\cdot-}$ state of reaction centers in which QFe was reduced prior to exposure to light, and that no triplet would be generated if I was also reduced prior to light activation, we titrated the attenuation of the ability of *Rps. viridis* chromatophores to generate a light induced triplet as a function of the ambient potential. An E_m value for $I/I^{\cdot-}$ in vitro of −400 mV at pH 10.8 with an *n* value of approximately 1 was obtained [19]. Subsequently, ps optical spectroscopy was used to measure the attenuation of the ability of isolated *Rps. viridis* reaction centers to generate the infrared absorbance band of $(BChl)_2^{\cdot+}$ at low redox potentials. The results suggested a similar E_m for $I/I^{\cdot-}$ in the isolated reaction center complex ($\approx$ −450 mV at pH 9.8) and lent support to the identification of I as the sole electron acceptor from photo-excited $(BChl)_2$ [13]. However, a recent paper by Klimov et al. [48] has reported an E_m value for $I/I^{\cdot-}$ of −620 mV in *Rps. viridis*. Because of this discrepancy, a re-examination of the thermodynamic properties of $I/I^{\cdot-}$ is included in the work presented here.

The major portion of the work in this paper was done in an attempt to clarify some of the puzzling spectroscopic properties of I. In particular we sought to determine the relationship, if any, between the two EPR signals and the two optical components attributable to $I^{\cdot-}$. In our previous papers [10–12] we have discussed whether the two EPR signals might arise from $I^{\cdot-}$ in two different environments, one sufficiently close to the $Q^{\cdot-}Fe$ (characterized by an EPR absorbance at *g* 1.82, refs. 24, 25) to interact magnetically with $Q^{\cdot-}Fe$ to give rise to the broad split EPR signal, while the other is magnetically isolated and gives rise to the free radical signal. There are two major possibilities in this model: first, $\mathrm{I}^{\cdot-}$ may involve another radical, such as $\mathrm{BChl}^{\cdot-}$ in addition to $\mathrm{BPh}^{\cdot-}$, in a situation where only one of the radicals is in a situation where it can magnetically interact with $\mathrm{Q}^{\cdot-}\mathrm{Fe}$. Alternatively, the single electron in $\mathrm{I}^{\cdot-}$ [10, 11] resides solely on $\mathrm{BPh}^{\cdot-}$, but only some of the reaction centers have $\mathrm{BPh}^{\cdot-}$ and $\mathrm{Q}^{\cdot-}\mathrm{Fe}$ properly positioned to allow magnetic interaction; in this case the optical changes of the 800 nm (830 nm) band would reflect secondary, perhaps electrochromic effects.

We have used chromatophores and reaction centers from *Rps. viridis* to examine these possibilities, and our results lend strong support to the suggestion [10, 11] that $\mathrm{I}^{\cdot-}$ and $\mathrm{Q}^{\cdot-}\mathrm{Fe}$ interact significantly, and that I is a single bacteriopheophytin molecule.

METHODS

Rps. viridis (NHTC 133) was grown photosynthetically, and photosynthetic membrane fragments (sometimes known as lamellae, but here referred to as chromatophores) were prepared as described previously [19]. Reaction center · cytochrome complexes were prepared using two different procedures: one used sodium dodecyl sulfate (SDS) and followed the method of Trosper et al. [16, 17] scaled up by a factor of 10–20; optical spectra of this preparation have been previously published [13, 16, 17]. The other method used lauryl dimethylamine *N*-oxide (LDAO) as the solubilizing agent, and details of this method are described below. *Rps. viridis* was grown in 20 l bottles illuminated by two quartzline lamps, in the medium described by Eimjhellen et al. [26]. The cells were stored at −20 °C until required.

Isolation of the LDAO-reaction center complex

The procedure is similar to that described recently by Pucheu et al. [27]. Tris · HCl, (50 mM, pH 8.0) buffer was added to 60 ml of packed cells to give a final volume of 500 ml, and the mixture was homogenized in a Waring blendor for 30 s. Cells in this suspension were broken by passage through a cell disrupter (Stanstead Fluid Power Ltd., Stanstead, Essex, U.K.) at 10 000 lbs./inch^2, and the eluate centrifuged at 20 000 × g for 5 min. The lightly colored supernatant was discarded and the pellet resuspended in 50 mM Tris · HCl buffer (final volume, 250 ml) by homogenization in a Waring blender. LDAO (a gift of Onyx Chemical Co., N.J.) (30 %) was added to the suspension to give a final concentration of 1 %, and the mixture was incubated for 1 h at 18–20 °C. After centrifugation at 35 000 × g for 10 min the supernatant was discarded, and the pellet resuspended as described above to give 100 ml of solution with an LDAO concentration of 1 %. Centrifugation of this incubated, LDAO-treated material yielded a green supernatant which was dialyzed overnight against 0.1 % LDAO. A DEAE-cellulose column (packed volume of 80 ml) was poured in a 100 ml glass syringe barrel and equilibrated with 50 mM Tris · HCl, pH 8.0. The dialyzed extract ($\lambda_{\max} \approx 685$ nm) was run into the column until the top one-third of the column was colored. The column was eluted with the buffer until the eluate, which was green (chlorin-containing) at first, ran colorless. The gray-brown reaction center-containing material was washed from the column with 10 mM sodium phosphate/150 mM sodium chloride, pH 7.0. Some chlorin (685 nm-absorbing) material was present and was removed either by chromatography on hydroxylapatite (see below) or by rerunning the eluate after diluting it 1 : 1 (v/v) with water on a new DEAE-cellulose column (20 ml packed volume). A hydroxylapatite [28] column (50 ml) was equilibrated with 10 mM sodium phosphate/200 mM sodium chloride, pH 7.0, and the reaction center and chlorin-containing eluate from the first DEAE-cellulose column was adsorbed to the hydroxylapatite. After washing the column with 50–100 ml of the equilibration buffer, the reaction center was eluated by 150 mM sodium phosphate/200 mM sodium chloride, pH 7.0. Immediately the P960-containing eluate was obtained free from the 685 nm contaminant, a saturated solution of ammonium sulfate was added until precipitation occurred (22–28 % ammonium sulfate). Centrifugation resulted in a floating precipitate which was collected on a sintered glass filter, and the precipitate was redissolved in 50 mM Tris, pH 8.0 and stored at −20 °C until needed. The SDS and LDAO-reaction centers both appear to contain 4 bacteriochlorophyll *b* and 2 bacteriopheophytin *b* molecules per reaction center [29].

Fig. 1 shows the optical spectra of the LDAO reaction centers as isolated (in the $[(BChl)_2I]QFe$ state) and of the same preparation when recorded in the light (i.e., in the $[(BChl)_2{}^{+}I]Q^{-}Fe$ state). Fig. 2 shows the difference between the two spectra recorded in Fig. 1. This difference spectrum also includes contributions from the light-induced oxidation of the *c*-type cytochromes associated with the reaction center; chromatophores possess two molecules of high potential cytochrome c_{558} (E_m at pH 7 = +346 mV, ref. 19) and two molecules of low potential cytochrome c_{553} ($E_{m,\,pH\,7}$ = +10 mV, [19]) tightly bound per reaction center (the subscripts referring

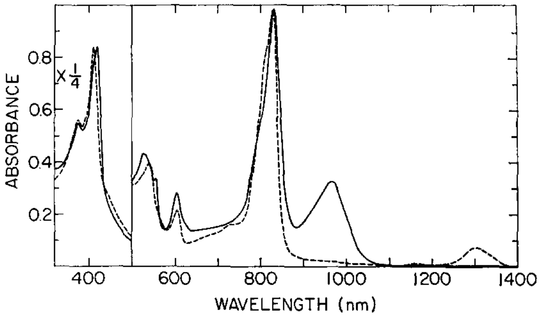

Fig. 1. Room temperature absorption spectra of *Rps. viridis* LDAO-reaction centers. The reaction centers (3 μM) were suspended in 50 mM Tris/Cl, pH 8.0, and the spectra were measured in a Cary 14R spectrophotometer in the 1R1 (solid line) or 1R2 (dashed line) modes.

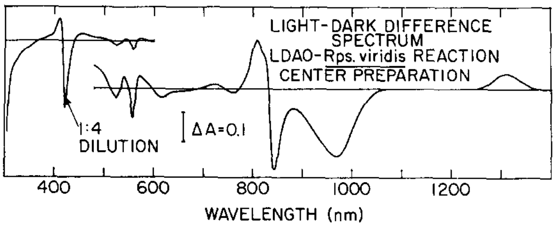

Fig. 2. Room temperature light minus dark difference spectrum of the *Rps. viridis* LDAO-reaction centers. The conditions were those of Fig. 1, except that the reaction center concentration was only 2 μM.

to their α-band absorbance maxima in the reduced form) and both the SDS- and LDAO-reaction centers appear to retain this cytochrome complement.

Optical and EPR properties of the reaction centers

The SDS- and LDAO-reaction centers are both free of the 680 nm chlorin contaminant of earlier preparations [33], and the two preparations are spectrophotometrically similar except that the 790 nm shoulder is more prominent in the SDS preparation, at both 300 K and 77 K. This is due to the presence of more of an 817 nm spectral component in the 830 nm peak of the LDAO preparation (ref. 29, cf. ref. 17). However, the EPR spectroscopic properties of the two reaction centers are quite distinct. Both reaction centers exhibit a light-induced spin polarized triplet signal (Fig. 3), with zero field splitting characteristics close to the values of $D = 157 \cdot 10^{-4}$ cm^{-1} and $E = 37 \cdot 10^{-4}\,\text{cm}^{-1}$ which we have measured in whole cells [19]. How-

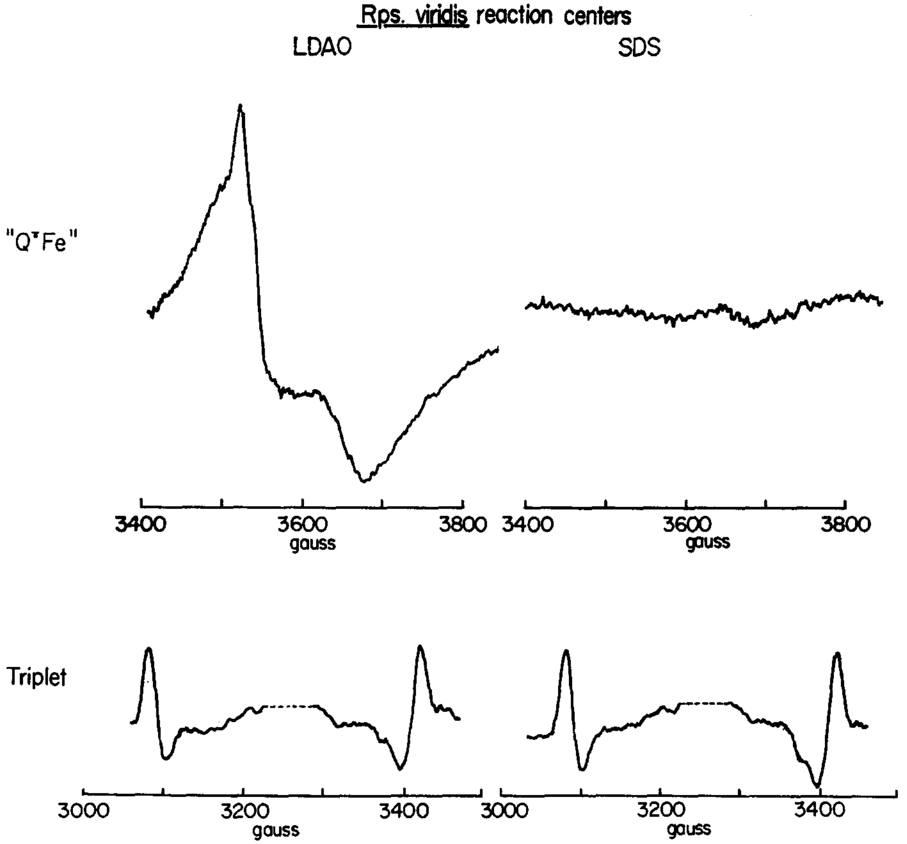

Fig. 3. Low temperature EPR spectra of the LDAO and SDS *Rps. viridis* reaction centers. The reaction centers (approx. 50 μM) were suspended in 50 mM Tris · HCl, pH 8.0, with a few crystals of sodium dithionite. Q^- Fe was measured at 9 K with 20 mW of microwave power, and a modulation amplitude of 25 G, while the triplet signals, which are light minus dark difference spectra were measured with 5 mW of microwave power and a modulation amplitude of 25 G at 6 K. The amplification of the signals was normalized so that the triplet signals were the same size.

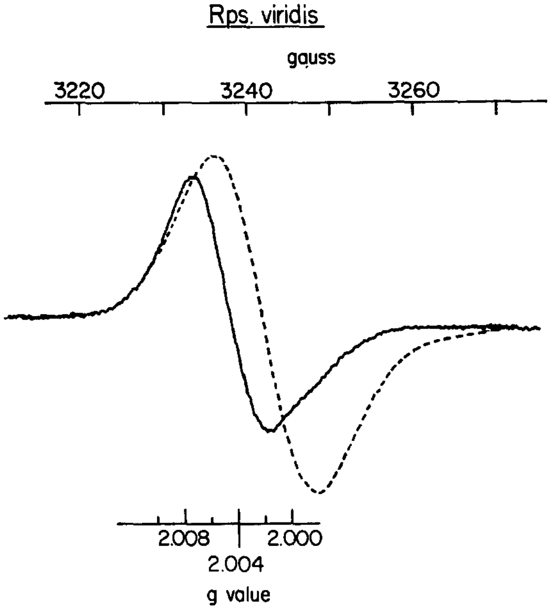

Fig. 4. Low temperature EPR spectra of the signals close to $g = 2$ in LDAO- and SDS-*Rps. viridis* reaction centers. The solid line represents the signal obtained with SDS-reaction centers frozen during illumination in the presence of sodium ascorbate to trap the primary acceptor in its reduced state. This was confirmed by analysis of the light-induced triplet signal as in Fig. 3. The dashed line represents the light induced $(BChl)_2^{+}$ signal in LDAO-reaction centers. The reaction centers (50 μM) were suspended in 50 mM Tris/Cl pH 8, and the spectra were measured at 8.5 K using 1 μW of microwave power and a modulation amplitude of 5 G.

ever, a major difference between the two reaction center preparations is seen in the EPR spectra of the primary acceptor QFe, as shown in Fig. 3. While the LDAO-reaction centers clearly show the g 1.82 signal of $Q^{\bar{\cdot}}Fe$, this signal is undetectable in the SDS-reaction centers (Fig. 3). However, the SDS-reaction centers possess a signal centered close to g 2.0045 with a peak to peak width of approximately 8 gauss (Fig. 4). This signal is very similar to that reported in reaction centers from *Rhodospirillum rubrum* [30] prepared using Triton X-100 at high pH, and in reaction centers from *Rhodopseudomonas sphaeroides* [34] treated with a mixture of SDS and LDAO. By comparing the signal in the SDS- and LDAO-treated reaction centers from *Rps. sphaeroides* with model compounds, Feher et al. [31] concluded that the g 2.0045 signal was due to a ubiquinone radical. *Rps. viridis* reaction centers apparently do not contain ubiquinone [27] but contain a menaquinone-like compound instead; we therefore tentatively assign the g 2.0045 signal shown in Fig. 4 to a free radical of this menaquinone-like substance. As we will discuss later, we believe that this difference in the EPR signals of the primary acceptor in the two reaction center preparations reflects the presence or absence of an interaction between the quinone-free radical and iron. Thus, in vivo the primary acceptor involves both quinone and iron (QFe), which in the reduced form is characterized by an EPR absorbance at g 1.82. This complex is apparently unaffected by LDAO (Fig. 3) but is apparently destroyed by SDS (Fig. 3 and ref. 31) perhaps by extracting the iron, as suggested by Feher et al. [31], or by moving the iron away from the quinone, or by changing its spin state.

The light-induced signal due to $(BChl)_2^{\dot{+}}$ can be readily observed in LDAO-reaction centers poised at an ambient potential to allow normal photochemistry (Fig. 4), and appears as a signal centered close to *g* 2.003 with a peak to peak width of approximately 12 gauss. This signal is indistinguishable in peak shape and line width from that observed in chromatophores [19, 32], presumably because all the signal due to the reduced primary acceptor appears at *g* 1.82 in both chromatophores and LDAO-reaction centers. In contrast, the light-induced signal in SDS-reaction centers (not shown) appears to be a mixture of the *g* 2.003 $(BChl)_2^{\dot{+}}$ signal plus the *g* 2.0045 $Q^{\dot{-}}$ signal. Since the latter signal is apparently more difficult to saturate with microwave power than the former, it is appropriate that the "central *g* value" of the composite light-induced signal can be slightly varied with the microwave power around *g* 2.0035.

Estimation of reaction center concentration. An assumed millimolar differential extinction coefficient for *P*-960 of 100 cm^{-1} at 965 nm was used [33].

Spectrometry. Optical spectra were obtained either with a Cary 14R spectrophotometer or the split-beam spectrophotometers used previously [11]. Electron paramagnetic resonance (EPR) spectra were obtained with Varian E-4 and E-9 spectrometers, with the samples cooled to cryogenic temperatures with a flowing helium cryostat [10, 11]. Light minus dark difference EPR spectra were obtained using a Nicolet 1074 computer. Redox potentiometry and the anaerobic transfer of samples to EPR tubes were as described previously [10, 11, 34].

RESULTS

Thermodynamic properties of I in Rps. viridis

As we have discussed in the Introduction, there is some controversy concerning the equilibrium E_m of $I/I^{\dot{-}}$ in *Rps. viridis*. We have measured a value of −400 mV at pH 10.8 in chromatophores [19] and of −450 mV at pH 9.8 in SDS-reaction centers [13], using redox potentiometry with viologen dyes as redox mediators. Klimov et al. [48] have also used redox potentiometry, but with neutral red as a redox mediator and have obtained values of −620 mV, between pH 8 and 11.5. There are several possibilities for errors in these estimations of the E_m of $I/I^{\dot{-}}$; for example, the added redox mediators might not truly equilibrate with I, or they might preferentially interact with only one form of the $I/I^{\dot{-}}$ redox couple, thereby altering its thermodynamic properties. We have, therefore, attempted to measure the E_m of the $I/I^{\dot{-}}$ couple in a manner which obviated the need for redox mediators, by comparing the E_m of the $I/I^{\dot{-}}$ couple with that of the hydrogen electrode. Such an approach has been successfully used by Lozier and Butler [35] to measure the E_m of the primary acceptor of chloroplast Photosystem I.

In these experiments, chromatophores were suspended in a variety of buffers at different pH values, and the redox potential was then lowered by the addition of several crystals of sodium dithionite. Control experiments in the presence of redox dyes and using electrodes have shown that under these conditions the ambient redox potential (E_h) drops to within ±30 mV of the E_m of the hydrogen ($1/2\ H_2/H^+$) couple at the specified pH (i.e., $E_0 - 60 \cdot \text{pH}$ mV, where E_0 is the standard potential of the hydrogen electrode, and equals 0 mV). However we found that the equilibration of the dithionite with I was very slow (its halftime appeared to be in the order of several hours), and that after these long periods of time at low redox potentials, some of the changes that had occurred were irreversible. To speed up the reaction we briefly illuminated the chromatophores in the presence of dithionite and then left them in the dark before freezing them for EPR analysis. The rationale for this was that the room temperature illumination would be expected to generate $I^{\cdot-}$, at least transiently, at all values of pH (see Fig. 13), while the subsequent dark period might allow $I^{\cdot-}$ to reoxidize if the ambient potential was more positive than the E_m of $I/I^{\cdot-}$. The amount of $I^{\cdot-}$ trapped at each pH would thus effectively compare the E_m of $I/I^{\cdot-}$ with that of the hydrogen electrode.

In our experiments, we illuminated the chromatophores in the presence of dithionite in EPR tubes for 1 min by placing them two inches from a 100 W tungsten filament lamp, during which time they were cooled by an electric fan. After illumination, the chromatophores were kept dark for 1 min, and then frozen in liquid nitrogen in the dark. The results are shown in Fig. 5. At pH 5, $I^{\cdot-}$ completely reoxidized in the dark period, and there was no detectable broad split signal present, and the light-induced triplet signal was as large as in unilluminated controls. Conversely, at pH 8.8 the broad split signal was as large as in control tubes which had received no dark relaxation time after the illumination and prior to freezing, while the light-induced triplet was undetectable in either tube. The "midpoint" of the trapping effect was

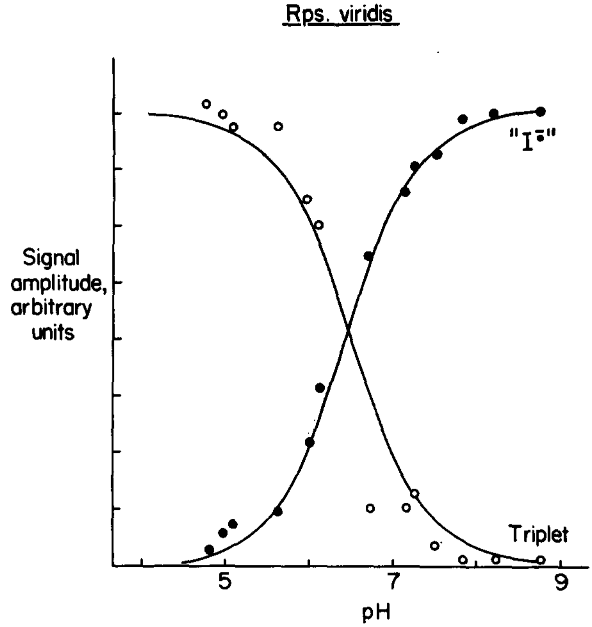

Fig. 5. Titration of the ability to photochemically trap I in its reduced state at room temperature. The experimental rationale is described in the text. Chromatophores (1 mM bacteriochlorophyll) were suspended in 100 mM KCl, 20 mM of an appropriate buffer, with a few crystals of sodium dithionite, illuminated for 1 min, left in the dark for 1 min, and then frozen at 77 K. The EPR analyses were performed at 8 K and 5 mW of microwave power with a modulation amplitude of 12.5 G. $I^{\cdot-}$ was measured as the height of the low field portion of the broad split signal shown in Fig. 6, but similar results were also obtained by measuring $I^{\cdot-}$ as the narrow free radical signal, also shown in Fig. 6.

approximately pH 6.5, at which value the hydrogen electrode has an E_m of -390 mV. If our experimental rationale is correct, and the I/$I^{\cdot -}$ couple is somehow in equilibrium with the hydrogen (1/2 H_2/H^+) couple, then Fig. 5 implies that the E_m of I/$I^{\cdot -}$ is -390 ± 30 mV, in remarkable agreement with the values we obtained previously with redox potentiometry [19, 13]. Of course, the experiment reported here can be criticized on several grounds, not least because light was required to reduce I, although this might simply be due to an inability of the highly charged reducing agent (dithionite) to interact with the protein-bound I. Nevertheless, these criticisms seem to be different from those that can be levelled at the values determined by redox potentiometry, and the remarkable agreement between the disparate approaches give us confidence that we have indeed measured the E_m of I/$I^{\cdot -}$ in *Rps. viridis* as approximately -400 mV.

The photochemical trapping of $I^{\cdot -}$

In our work with *C. vinosum* we developed a method for trapping I^- at low temperatures. This involved poising reaction centers at an ambient potential where QFe was reduced prior to illumination, freezing, illuminating the reaction centers for 3 min at 200 K, and then subsequent storage of the samples at 77 K. This procedure allowed $I^{\cdot -}$ to be trapped on a days time-scale [10–12]. Other workers have used warmer temperatures; Shuvalov and Klimov [15] did most of their work at room temperature, and under these conditions the "half life" of $I^{\cdot -}$ in the dark was only 5 s: van Grondelle et al. [14] used -15 °C, and the "half life" was then several minutes. Presumably, the trapping of $I^{\cdot -}$ at all temperatures involves the concomitant oxidation of cytochrome *c*, but this has only been clearly demonstrated in the low temperature experiments, because at room temperatures the oxidized cytochrome is promptly reduced by the low ambient redox potential. A careful quantitation of the amount of *c*-type cytochrome oxidized during I reduction at 200 K revealed that only one electron was involved in this trapped $I^{\cdot -}$ state [10, 11] but such quantitation is very difficult at warmer temperatures because of the rapid rereduction of the cytochrome, and it is possible that the room temperature reduction of $I^{\cdot -}$ may involve more than one electron, which might drastically change the EPR properties of the trapped $I^{\cdot -}$ state. We have, therefore, examined the spectroscopic properties of $I^{\cdot -}$ in *Rps. viridis* when it is trapped at 200 K using the method of Tiede et al. [10, 11] or at room temperature, using the procedure outlined above for Fig. 5 (cf. refs. 14–18). For convenience we will refer to the methods as 200 and 300 K illuminations. Because we were also interested in the possible interaction between $I^{\cdot -}$ and $Q^{\cdot -}$Fe, we have examined the effects of these two illumination procedures on chromatophores and LDAO-reaction centers, which possess the *g* 1.82 signal, and SDS-reaction centers, which do not (Fig. 3). The results are presented below.

A. The trapping of $I^{\cdot -}$ *in chromatophores at 200 K.* Unlike the situation in *C. vinosum* [10, 11] the trapping of $I^{\cdot -}$ at 200 K in chromatophores of *Rps. viridis* generates only a single EPR signal (Fig. 6). This broad, split signal has similar properties to the analagous signal in *C. vinosum* [10, 11]; it is difficult to saturate with microwave power, and is only detected at temperatures below 20 K. However, it shows additional structure which was not discerned in the *C. vinosum* spectra [10, 11]; there is a positive shoulder on the low field side of the signal, and a more prominent negative shoulder on the high field side of the signal. Such structure suggests that there is some dipolar interaction between $I^{\cdot -}$ and some other paramagnetic species, perhaps $Q^{\cdot -}$Fe,

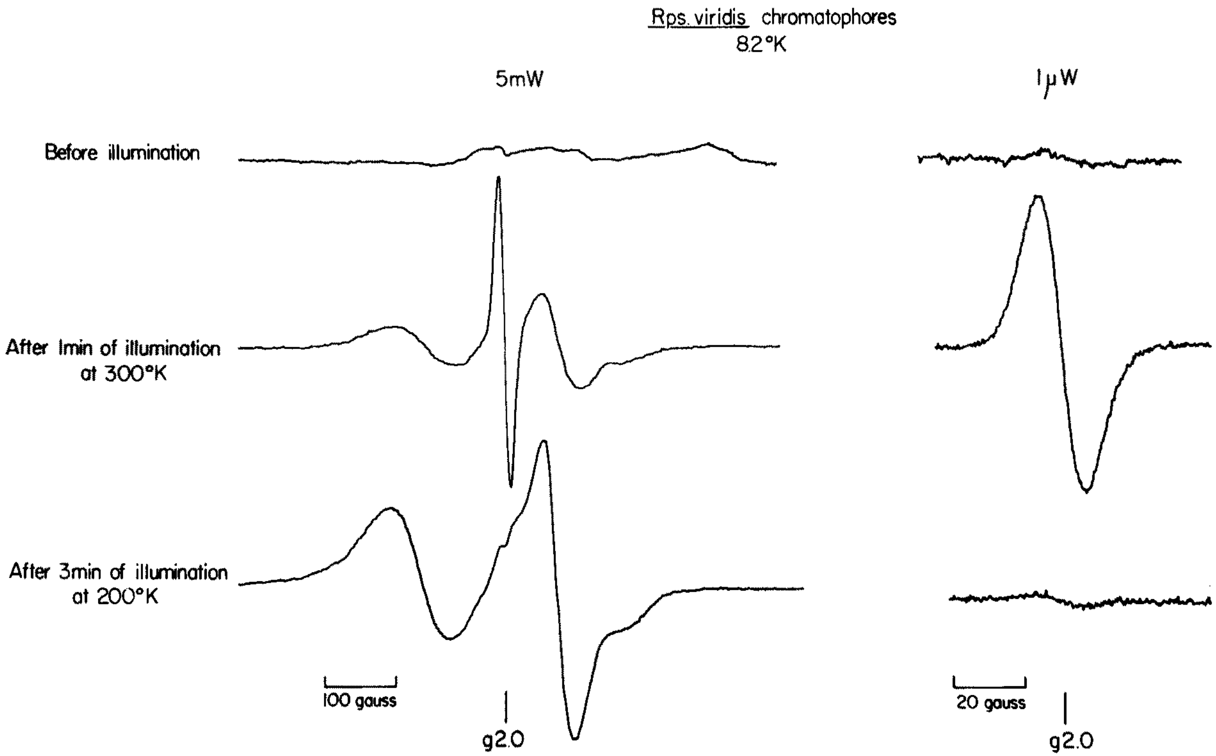

Fig. 6. EPR spectra of $I^{\cdot-}$ trapped at 200 and 300 K in chromatophores of *Rps. viridis*. Chromatophores (1.2 mM bacteriochlorophyll) were suspended in 100 mM KCl, 20 mM *N*-Morpholino propane sulfonic acid, pH 7.5 with a few crystals of sodium dithionite. One tube was illuminated at room temperature as in Fig. 5, another was frozen to 200 K and illuminated at that temperature for 3 min before cooling to 77 K, while a third was frozen to 77 K without any illumination. The EPR spectra were measured at 8.2 K with either 5 mW of microwave power and 12.5 G modulation amplitude or with 1 μW of microwave power and a modulation amplitude of 5 G.

and if this explanation is correct we would expect some effects on the EPR signal of the dipolar partner.

The 200 K trapping of $\mathrm{I}^{\overline{\cdot}}$ indeed had a dramatic effect on the *g* 1.82 signal; it apparently disappeared! We therefore examined the reversibility of this disappearance to ensure that it was not caused by irreversible damage incurred during the trapping of $\mathrm{I}^{\overline{\cdot}}$. Our earlier work [19] indicated midpoint potentials for the primary acceptor QFe/$\mathrm{Q}^{\overline{\cdot}}$Fe and intermediate I/$\mathrm{I}^{\overline{\cdot}}$ couples of ***Rps. viridis*** as −150 mV and −400 mV, respectively. We therefore expected that 200 K illumination of samples poised at redox potentials intermediate between these two values should trap I in its reduced state, but that $\mathrm{I}^{\overline{\cdot}}$ would reoxidize (and cytochrome c_{553} rereduce) if the sample were warmed to 300 K and then refrozen. Fig. 7 shows this to be the case for a sample

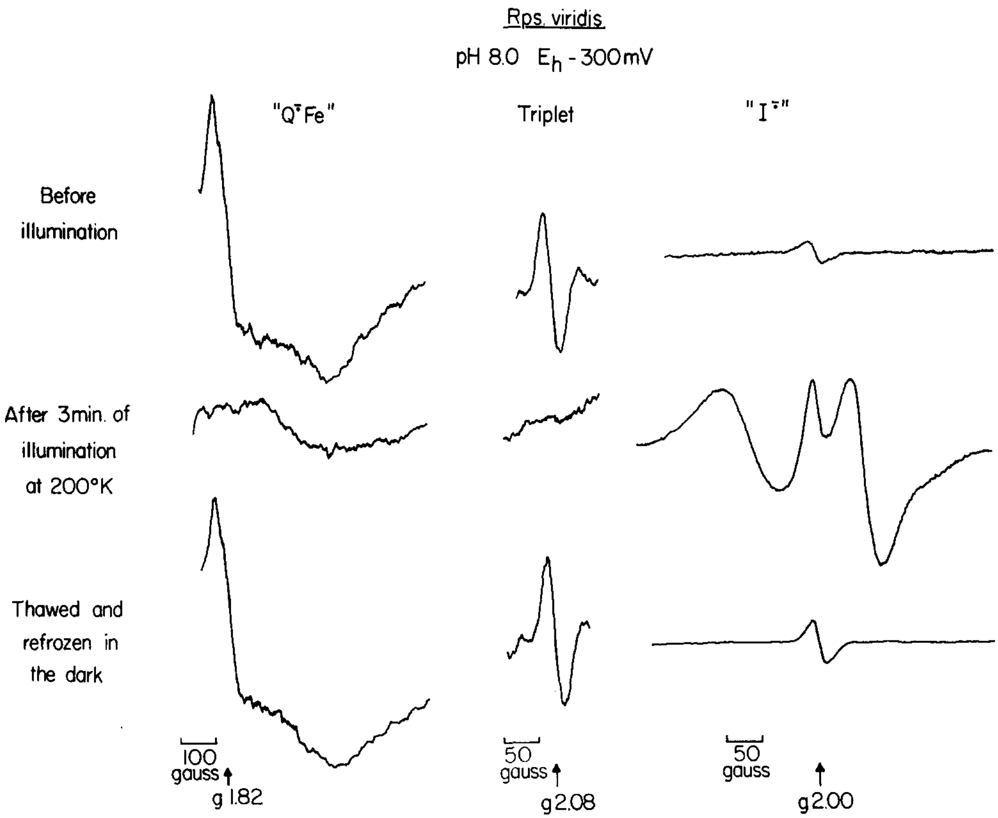

Fig. 7. The reversible generation of $\mathrm{I}^{\overline{\cdot}}$ in *Rps. viridis* chromatophores. Chromatophores (1 μM bacteriochlorophyll) were suspended in 100 mM KCl, 20 mM Tris/Cl pH 8.0, with 50 μM each of *N*-methyl phenazine methosulfate, pyocyanine, methyl viologen and benzyl viologen as redox mediators. The ambient potential was adjusted to −300 mV with sodium dithionite and a sample was then frozen in the dark to 77 K. The EPR properties of the sample were measured as shown, and the sample was then warmed to 200 K, illuminated for 3 min, and recooled for EPR analysis. Finally the sample was thawed, and refrozen in the dark for EPR analysis. All the EPR analysis was performed at 6 K with the following instrument settings; the low field "wing" of the light minus dark triplet signal at 5 mW microwave power and 25 G modulation amplitude, $\mathrm{Q}^{\overline{\cdot}}$Fe at 20 mW and 20 G, and $\mathrm{I}^{\overline{\cdot}}$ at 5 mW and 12.5 G.

poised at E_h −300 mV at pH 7. As expected from our previous work, the ability to generate a light-induced triplet or biradical signal requires I in its oxidized state, and the generation of $I^{\cdot -}$ prevented light-induced triplet formation, while the subsequent reoxidation of $I^{\cdot -}$ resulted in the return of the light-induced triplet signal to its original magnitude. The apparent disappearance of the *g* 1.82 signal concommitant with the reduction of I was also reversed by the reoxidation of $I^{\cdot -}$.

B. The trapping of $I^{\cdot -}$ in chromatophores at 300 K. The 300 K illumination generates EPR signals (Fig. 6) which are qualitively similar to those seen in *C. vinosum* [10, 11], involving a narrow free radical signal centered close to *g* 2.003, which is relatively easy to saturate with microwave power, together with the broad asymmetrically split signal seen after the 200 K illumination discussed above. At low microwave power (right hand side of Fig. 6) the broad signal is not detected and only the narrow signal is resolved; it is centered close to *g* 2.003, and has a peak to peak width of approximately 13 gauss.

Trapping $I^{\cdot -}$ with 300 K illumination also caused the disappearance of both the light-induced triplet signal and the *g* 1.82 signal, and again the reoxidation of $I^{\cdot -}$ restored both signals to their former level.

C. The trapping of $I^{\cdot -}$ in LDAO-reaction centers at 200 K. The EPR signals associated with trapping $I^{\cdot -}$ at 200 K in LDAO-reaction centers from *Rps. viridis* are shown in Fig. 8; like the signals generated in *C. vinosum* reaction centers at 200 K [10–12] and *Rps. viridis* chromatophores at 300 K (Fig. 6), there are two distinct

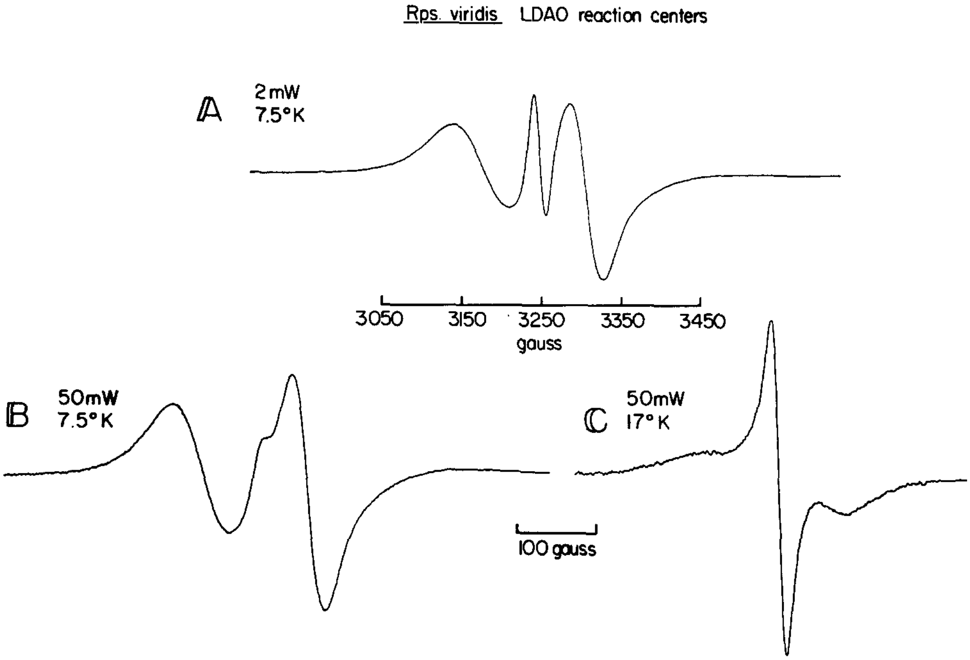

Fig. 8. EPR signals associated with $I^{\cdot -}$ in *Rps. viridis* LDAO-reaction centers. The reaction centers used in Fig. 3 were illuminated for 3 min at 200 K and were then cooled to 77 K. The EPR spectra were run at the microwave powers and temperatures indicated, using a modulation amplitude of 10 G.

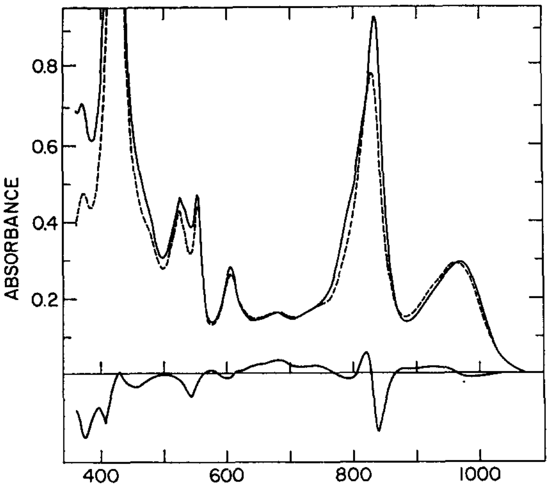

Fig. 9. Room temperature absorption spectra of *Rps. viridis* LDAO-reaction centers at low redox potential. The reaction centers (3 μM) were suspended as in Fig. 1 in the presence of a few crystals of sodium dithionite, and the spectra were recorded in the 1R1 (solid line) and 1R2 (dashed line) modes of the Cary 14R spectrophotometer. Underneath is the difference between these two spectra.

EPR signals which can be resolved by varying the conditions of measurement (Fig. 8). Just as in the chromatophore case (Fig. 7), the trapping of $I^{\cdot -}$ is accompanied by the reversible disappearance of both the *g* 1.82 signal of $Q^{\cdot -}$Fe, and the ability to generate the light-induced spin polarized triplet.

The optical changes associated with the trapping of $I^{\cdot -}$ at 200 K in LDAO-reaction centers were similar to those shown in Fig. 9.

D. The trapping of $I^{\cdot -}$ in LDAO-reaction centers at 300 K. Fig. 9 shows the

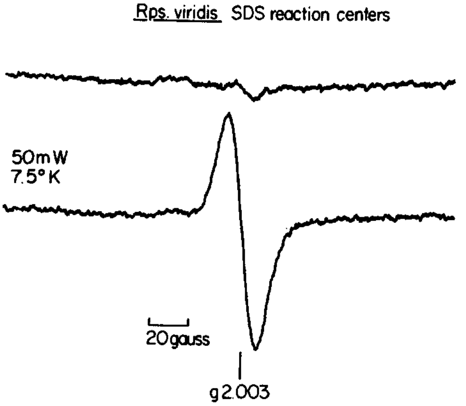

Fig. 10. The EPR signal associated with $I^{\cdot -}$ in *Rps. viridis* SDS-reaction centers. The reaction centers used in Fig. 3 were illuminated for 3 min at 200 K, and were then cooled to 77 K. The EPR spectra were run at 7.5 K using 50 mW of microwave power, and a modulation amplitude of 5 G. Such conditions partially saturate the $I^{\cdot -}$ signal, but should optimise any broad split EPR signal (cf. Fig. 8B). Such conditions also dramatically saturate the free radical signal attributed to the semiquinone primary acceptor signal of Fig. 4.

TABLE I

SPECTROSCOPIC PROPERTIES OF $I^{\cdot -}$ IN *RPS. VIRIDIS* PREPARATIONS.

This table summarizes the data of Figs. 6–10. The first column represents the presence or absence of the *g* 1.82 $Q^{\cdot -}Fe$ signal in the preparation before the trapping of $I^{\cdot -}$, while the remainder represent the EPR and optical properties of $I^{\cdot -}$ trapped in the various preparations at the stated temperature. In all cases + signifies presence, 0 signifies absence, and – indicates that the optical properties of $I^{\cdot -}$ have not been measured in chromatophores because of the high background absorbance of the antenna pigments.

	$Q^{\cdot -}Fe$	Temperature of photoreduction of I	After trapping $I^{\cdot -}$		EPR properties of $I^{\cdot -}$		Optical properties of $I^{\cdot -}$		
	g 1.82		*g* 1.82	Triplet (hν at 7 K)	Broad split signal	Free radical	540 nm bleaching	830 nm "shift"	960 nm "shift"
Chromatophores	+	200	0	0	++++	0	–	–	–
		300	0	0	+++	+	–	–	–
LDAO-reaction centers	+	200	0	0	+++	+	+	+	+
		300	0	0	++	++	+	+	+
SDS-reaction centers	0	200	0	0	0	++++	+	+	+
		300	0	0	0	++++	+	+	+

optical changes induced by trapping I^{-} at 300 K in LDAO-reaction centers. The changes are very similar to those reported in SDS-reaction centers by Trosper et al. [16, 17] and Shuvalov et al. [18], and appear to involve a bleaching of the bacteriopheophytin *b* at 540 and 790 nm, together with a prominent blue shift of the 830 nm band and a less pronounced blue shift of the 960 nm band. The EPR signals induced by this treatment were similar to those of Fig. 8, except that the proportions of the two signals were different, and the unsplit free radical greatly predominated.

E. The trapping of I^{-} in SDS-reaction centers at 200 K. Trapping I^{-} at 200 K in SDS-reaction centers generated the EPR signal shown in Fig. 10; there is only a single narrow free radical, with properties very similar to the free radical produced by 300 K illumination of chromatophores. No broad split signal could be detected, even at the low temperatures and high microwave powers which maximize this signal (Fig. 10). Nevertheless, the light-induced triplet could not be detected after the 200 K illumination of the SDS-reaction centers, indicating that the reduction of I^{-} was complete.

The optical changes induced by this low temperature illumination were similar to those measured after illumination at 77 K [13] or 300 K [16–18].

F. The trapping of I^{-} in SDS-reaction centers at 300 K. The optical changes induced by this method of reducing I^{-} have already been discussed by Trosper et al. [16, 17] and Shuvalov et al. [18]; the EPR signal produced was identical to that in Fig. 10.

Summary of the data

We have tabulated all the findings described above into Table I. In all cases the

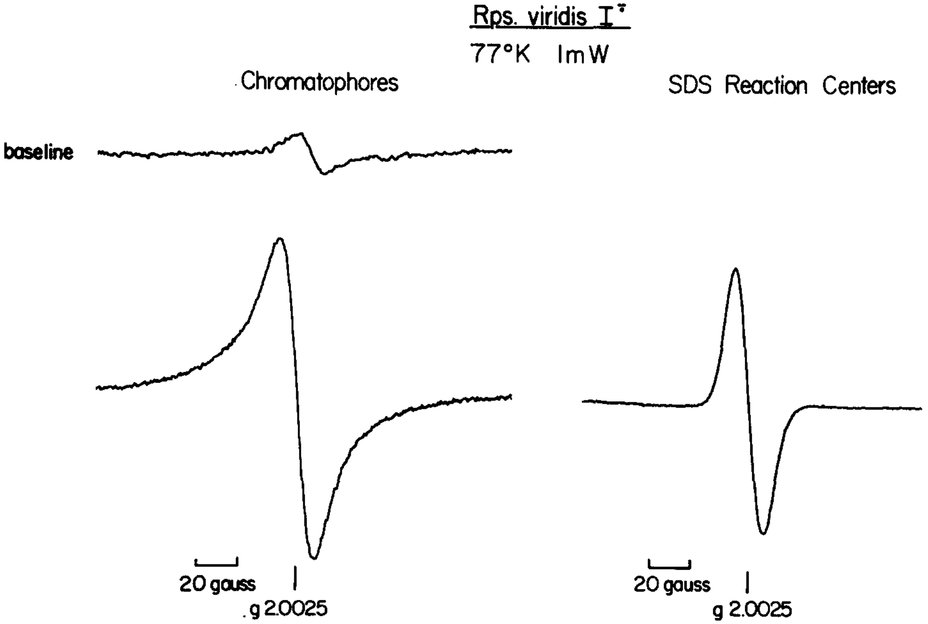

Fig. 11. The EPR spectra of I^{-} at warmer temperatures in *Rps. viridis* chromatophores and SDS-reaction centers. The samples used were the chromatophores illuminated at 200 K in Fig. 6, and the SDS-reaction centers of Fig. 10 and the spectra were measured at 77 K using 1 mW of microwave power and a modulation amplitude of 5 G.

EPR changes were almost completely reversible (> 95 %). However, as discussed by Trosper et al. [16, 17], illuminating isolated reaction centers in the presence of dithionite caused some irreversible optical changes, most notably in the 830 and 680 nm bands; this was attributed to the photobleaching of contaminating chromophores and the degradation of a small proportion of the reaction centers. In order to avoid contributions from such effects, Fig. 9, and the data in Table I, reflect only reversible optical changes.

The EPR characteristics of I$^{\cdot-}$ at warmer temperatures. As we have mentioned above, the broad split signal associated with I$^{\cdot-}$ in chromatophores and LDAO-reaction centers is very temperature sensitive and is difficult to detect above 15 K. However, as the broad split signal disappears as the temperature is raised, it is replaced by a signal centered close to *g* 2.003 (Fig. 11). This signal, which is also difficult to saturate with microwave power, is rather broad, with a peak to peak width of approximately 17 G; its line shape approximates a lorentzian curve. The I$^{\cdot-}$ signal of the SDS-reaction centers is quite different; its shape does not apparently vary with temperature, and at 77 K it is still gaussian in lineshape with a peak to peak width of 13 G centered close to *g* 2.003 and it remains readily saturable. The I$^{\cdot-}$ signal in LDAO-reaction centers at 77 K is apparently a mixture of the two signals shown in Fig. 11.

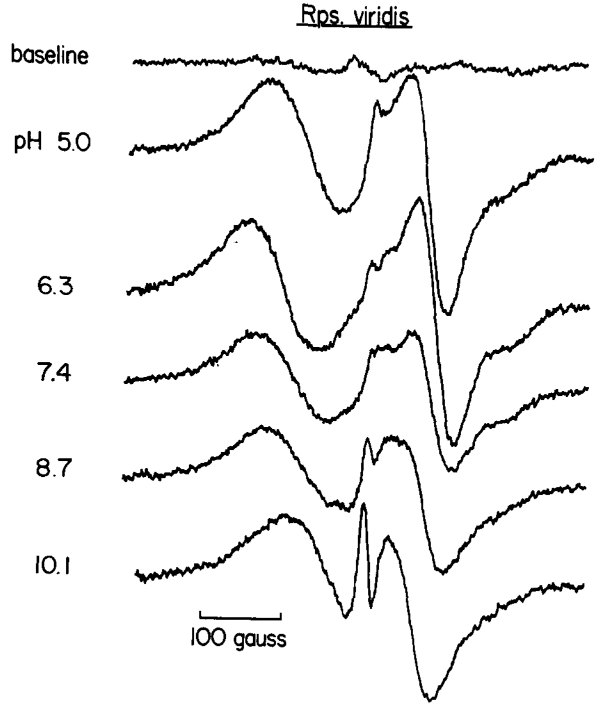

Fig. 12. The effect of pH on the EPR signals of I$^{\cdot-}$ in *Rps. viridis* chromatophores. Chromatophores (1 mM bacteriochlorophyll) were suspended in 100 mM KCl, 20 mM of an appropriate buffer with a few crystals of sodium dithionite, frozen to 200 K, illuminated for 3 min at this temperature, and then cooled to 77 K until EPR analysis. The spectra were obtained at 6.7 K with 2 mW of microwave power and a modulation amplitude of 5 G.

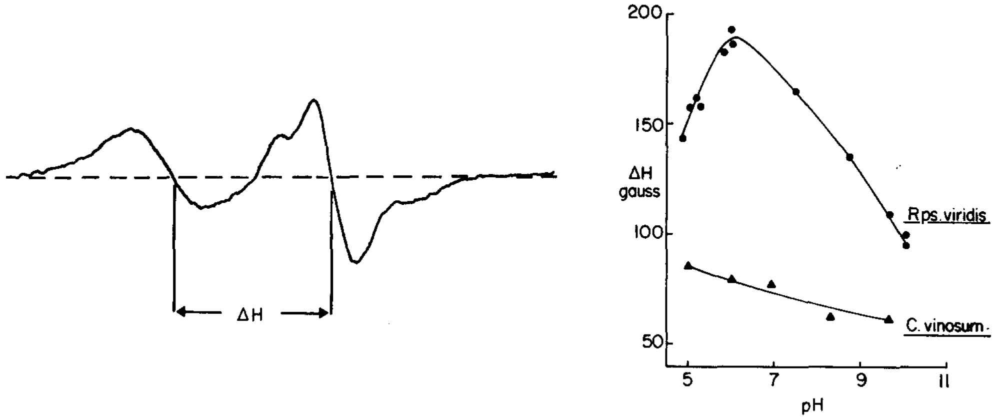

Fig. 13. The effect of pH on the splitting of the broad split signal in chromatophores of *Rps. viridis* and *C. vinosum*. The splitting of the broad signal, ΔH, was measured as shown at a variety of different values of pH, using the conditions of Fig. 12. Chromatophores of *C. vinosum* (1.4 mM bacteriochlorophyll) were similarly treated, and measured with similar spectrometer conditions.

The pH dependency of the lineshape of $I^{\cdot -}$. During the course of the experiments reported above, we found that the splitting of the broad split signal varied with pH. Fig. 12 shows the results of a formal experiment to examine this phenomenon, and the results are presented graphically in Fig. 13. In *Rps. viridis* the splitting is much wider than that previously observed in *C. vinosum* [10, 11], and is maximal at pH 6, where the splitting is almost 200 G. The splitting observed in *C. vinosum*, which is of the order of 60 G at neutral pH [10, 11] is essentially independent of pH (Fig. 13).

DISCUSSION

The traditional "primary acceptor" of Rps. viridis

The chemical identity of the traditional "primary acceptor" (which has often been known as X) has been the subject of some controversy in the past, but there is now a general consensus that the most likely candidate is a quinone · iron complex (abbreviated to QFe) where the electron from the primary donor resides mainly on the quinone [36]. The EPR signal of this $Q^{\cdot -}$Fe complex is usually characterized by an absorbance at *g* 1.82 (refs. 37–39, 19 and Figs. 6 and 9), but under conditions where iron has been at least partially extracted the spectrum is replaced by a much narrower free radical signal close to *g* 2.0045 [30, 31] which has been identified as a quinone-free radical. The methods for removing the iron have involved the use of either Triton X-100 as a detergent at alkaline pH (in *R. rubrum*, ref. 30) or a mixture of SDS and LDAO as the detergent (in *Rps. sphaeroides*, ref. 31). A comparison of the EPR spectrum of the "primary acceptor" in the two different reaction center preparations from *Rps. viridis* used here shows that it appears as a *g* 1.82 signal in LDAO-reaction centers (Fig. 3) and as a *g* 2.0045 signal in SDS-reaction centers (Fig. 4). We interpret this as indicating that the SDS treatment has stopped the interaction of the quinone with the iron, either by extracting the iron [30, 31] or perhaps by merely moving it or changing its spin-state [31]. The analysis of iron in reaction centers from *Rps. viridis* will require a high level of accuracy because of the presence of *c*-type cytochromes which would be expected to contribute four irons per reaction center [17, 19].

Extraction and reconstitution studies with *Rps. sphaeroides* reaction centers have provided strong evidence that in this species the quinone of QFe is ubiquinone [40, 41], while menaquinone apparently fulfills this role in *C. vinosum* [42]. *Rps. viridis* contains both ubiquinone and menaquinone [43] but only the latter is associated with the reaction center [25]. We therefore conclude that the "primary acceptor" of *Rps. viridis* is a menaquinone · iron complex under physiological conditions, which in the reduced form is characterized by an EPR absorption at *g* 1.82 [19]. The complex is apparently destroyed by SDS, so that the menaquinone-free radical now appears at *g* 2.0045, undisturbed by the iron. The use of LDAO does not appear to affect the QFe interaction so drastically and the reduced form still absorbs at *g* 1.82.

The intermediary carrier I: optical properties

The optical changes accompanying the reduction of I are qualitatively similar in the LDAO-reaction centers (Fig. 9) to those measured in SDS-reaction centers by Trosper et al. [16, 17], Shuvalov et al. [18] and ourselves [13]. There is a bleaching at 790 and 545 nm, together with apparent shifts of the 830 nm and 960 nm bands. We attribute the bleaching of the 790 and 545 nm bands, and the appearance of a broad band at 680 nm, to the reduction of bacteriopheophytin to its radical anion, $BPh^{\cdot-}$ [16–18, 44, 45]. The changes in the 830 and 960 nm bands are more difficult to interpret, although perhaps the simplest explanation is that the 830 nm bacteriochlorophyll(s) *b* undergoes a blue-shift in response to the reduction of bacteriopheophytin. This possibility has been suggested before [10–18], but it is possible that there is also a bleaching of the 830 nm region. In *C. vinosum* we discussed the possibility that the analogous change at 800 nm might reflect the presence of some reduced bacteriochlorophyll in the $I^{\cdot-}$ state [11] although we also considered, as did van Grondelle et al. [14], the possibility that the bleaching reflected a shift of an absorption band at 800 nm to a somewhat broader band with a maximum at 785 nm. The optical changes associated with the reduction of $I^{\cdot-}$ in SDS-reaction centers from *Rps. viridis*, measured at 77 K, appeared to show only a blue shift of the 830 nm band, with very little bleaching [13]; however, at room temperature the difference spectra (refs. 16–18 and Fig. 9) are more like those seen [11] in *C. vinosum*. We note that a somewhat similar blue shift of the 830 nm band occurs in response to the state $[(BChl)_2^{\cdot+}\ I]QFe^{\cdot-}$ (Fig. 2). The analogous shift of the 800 nm band in *Rps. sphaeroides* is now believed to be due to the bleaching of a 810 nm spectral component of $(BChl)_2$ and the appearance at 790 nm of the absorbance of $(BChl)_2^{\cdot+}$ [46], whereas the 830 nm blue shift in *Rps. viridis* has recently been shown [16, 17] to be the resultant of the blue shift of two spectral components, 836 and 846 nm, one of which may be equivalent to the 810 nm component of $(BChl)_2$ in *Rps. sphaeroides*. It is not unlikely that the changed electronic configurations in the $[(BChl)_2^{\cdot+}\ I]Q^{\cdot-}Fe$ and $[(BChl)_2\ I^{\cdot-}]Q^{\cdot-}Fe$ states could both result in an identical, or very similar, peak shift of one of the closely associated 830 nm bacteriochlorophyll molecules. However, linear dichroism of the 830 nm shift in *Rps. viridis* (Fig. 9 and refs. 16–18) and the 800 nm shift in *C. vinosum* and *C. minutissimum* [10–15] need to be studied before any firm explanation can be offered about the absorbance changes occurring around 830 nm when $I \rightarrow I^{\cdot-}$. The 960 nm shift in *Rps. viridis* (Fig. 9 and refs. 16–18) and the 870 shift in the *Chromatium* species [10–15] may also reflect an electrochromic shift induced by the presence of the unpaired electron on $I^{\cdot-}$, but these effects also deserve more sophisticated optical analysis.

Nevertheless, one important conclusion from the work presented here is that the optical changes accompanying the reduction of I, whatever they represent, are similar in both the LDAO- and SDS-reaction centers (Table I), despite the marked dissimilarity in the EPR spectra associated with $I^{\cdot-}$ in the two systems.

The intermediary carrier I: EPR properties

The EPR spectra associated with $I^{\cdot-}$ in *Rps. viridis* chromatophores are in some ways simpler than those obtained with *C. vinosum* [10–12]. As shown in Figs. 6, 7, and 13, the 200 K trapping of $I^{\cdot-}$ yields EPR spectra consisting entirely of a broad split signal, with no unsplit free radical signal present, at least at neutral pH. However, the splitting of the signal is much greater than that observed in *C. vinosum* (Fig. 13 and refs. 10–12), and shows additional structure that could not be resolved in the *C. vinosum* spectra [10–12]. In *Rps. viridis* there are additional "wings" on the split signal; the negative wing on the high field side of the signal is more prominent than its low field partner, but both can be resolved by assuming that the predominant split signal is composed of two gaussian curves and subtracting them from the observed spectrum. Interestingly, the splitting between the low field maximum and the high field minimum is approximately twice the splitting (ΔH in Fig. 13) between the two major bands. We have already discussed the split EPR signal in terms of an interaction between $I^{\cdot-}$ and $Q^{\cdot-}Fe$ [10–12], but because we could not resolve additional wings in *C. vinosum* we considered this mainly in terms of an exchange coupling: the resolution of the "wings" in *Rps. viridis* suggests that the interaction also involves dipolar coupling. The proposed dipolar splitting of the EPR signals associated with $I^{\cdot-}$ can also be seen in the LDAO-reaction centers (Fig. 8), although this is less prominent than in the chromatophore system. Nevertheless, substraction of gaussian signals from Fig. 8B reveals two extra "wings" separated by approximately twice the splitting of the major gaussian bands, and similar manipulations of the *C. vinosum* spectra [10–12] suggest that dipolar interactions may occur in this species as well.

The role of QFe. We are still left with the problem of the identity of the species interacting with $I^{\cdot-}$ to cause the broad split signal. In our earlier work we suggested that the $Q^{\cdot-}Fe$ might fulfill this role [10–12], and indeed the long-known observation that the photoreduction of QFe results in a red shift of the absorption bands of bacteriopheophytin [50–52] can now be interpreted in support of this idea. Further support for the involvement of $Q^{\cdot-}Fe$ in the broad split EPR signal comes from the reaction center studies reported here. The SDS-reaction centers, which possess no *g* 1.82 (Fig. 3) exhibit an $I^{\cdot-}$ EPR spectrum which is solely an unsplit, relatively easily saturated free radical (Fig. 10). The LDAO-reaction centers, which contain substantial amounts of the *g* 1.82 signal (Fig. 4) exhibit $I^{\cdot-}$ EPR spectra composed of both an unsplit radical plus the broad split signal seen in chromatophores (Fig. 8), while chromatophores, which presumably possess maximal amounts of the *g* 1.82 signal under optimal conditions, show no unsplit $I^{\cdot-}$ EPR signal (Fig. 6). There is, thus, a correlation between the presence of the *g* 1.82 signal and the ability to observe $I^{\cdot-}$ as the broad split signal, which leads us to believe that the presence of any unsplit free radical in $I^{\cdot-}$ reflects some form of destruction or physical movement of at least a portion of the QFe *g* 1.82 signal. This would explain the appearance of a small amount of unsplit signal at the more extreme values of pH in Fig. 12, and in Fig. 7 where the chromatophores had been incubated anaerobically for approximately 2 h while the redox potential was allowed to equilibrate. Our work with *C. vinosum* [11] and similar studies on *Rps. viridis* indicated that the photochemical reduction of $I^{\cdot-}$ at 200 K involves only a single electron, but we have been unable to determine the number of electrons involved in the 300 K trapping of $I^{\cdot-}$ because any photooxidized cytochrome is promptly rereduced. If more than one electron is involved at 300 K, an additional explanation for the presence of unsplit $I^{\cdot-}$ could be the double reduction of the paramagnetic $Q^{\cdot-}$Fe to the diamagnetic Q^{2-}Fe, which could not interact with $I^{\cdot-}$. However, this cannot explain the presence of both split and unsplit signals in the 200 K $I^{\cdot-}$ state in whole cells of *C. vinosum* [10, 11], so in this case we assume that only part of $I^{\cdot-}$ is close enough to $Q^{\cdot-}$Fe to magnetically interact, even under physiological conditions.

A somewhat unexpected finding, shown in Fig. 7, is that the *g* 1.82 $Q^{\cdot-}$Fe signal apparently disappears concommitantly with I reduction. This might represent a second reduction of the $Q^{\cdot-}$Fe as discussed above, but this cannot be the explanation for the apparent disappearance at 200 K, for if this occurred there would be no paramagnetic species available to interact with I to yield the broad split EPR signal of Figs. 6–8. Alternatively, the EPR signal at *g* 1.82 might be split and broadened by its interaction with $I^{\cdot-}$ so that it lost its prominent features. This latter explanation seems more likely, particularly since simulations involving splitting the signal by the same ΔH as that exhibited by $I^{\cdot-}$ to model the postulated exchange interaction, with additional broadening of the split signals to model the dipolar interaction with $I^{\cdot-}$, readily mimic the apparent disappearance of the *g* 1.82 signal shown in Fig. 7.

Further evidence that QFe is exchange coupled to $I^{\cdot-}$ comes from the temperature-dependence of the broad split $I^{\cdot-}$ EPR signal. As we found in *C. vinosum* [11], the two centers have similar relaxation times, as indicated by their similar, and rather unusual temperature/power dependencies. While a detailed analysis of this phenomenon will be described in a separate paper, it is noteworthy that at temperatures above 20 K, where neither the *g* 1.82 $Q^{\cdot-}$Fe signal nor the broad split $I^{\cdot-}$ signal can be detected, $I^{\cdot-}$ is seen as a broad lorentzian line centered close to *g* 2.003 (Fig. 11). This might be expected if $I^{\cdot-}$ were exchange coupled to a very rapidly relaxing paramagnetic system, for which a prime candidate would be one involving a metal iron, such as QFe. Support for this conjecture comes from the SDS-reaction centers which lack the *g* 1.82 QFe EPR signal (Fig. 3) and do not show a lorentzian $I^{\cdot-}$ at 77 K (Fig. 11).

The effect of pH on $I^{\cdot-}$. We examined the pH dependency of the splitting of the broad signal because we had previously identified a p*K* on QFe ($Q^{\cdot}$HFe/$Q^{\cdot-}$Fe) at pH 7.8 [19] and we wished to test the possibility that the state of protonation of QFe might affect its interaction with $I^{\cdot-}$. Figs. 12 and 13 show that there is a dramatic effect of pH on ΔH in *Rps. viridis* but the effect does not seem to be related to the p*K* on the QFe. In this respect it is noteworthy that ΔH in *C. vinosum* is essentially independent of pH, despite the fact that QFe has a very similar p*K* in this species [47]. We thus ascribe the pH-dependent splitting of $I^{\cdot-}$ to some general effect of pH on the reaction center which perhaps alters the spatial arrangement of I and QFe. Picosecond experiments are currently underway to determine whether the change in interaction between QFe and $I^{\cdot-}$ at different values of pH has any dramatic effect on the rate of electron transport between these two components during normal photochemistry.

The intermediary carrier I: its equilibrium midpoint potential

The experiments of Fig. 5 appear to confirm our earlier redox titrations of the I/I$^{\cdot-}$ couple in *Rps. viridis* chromatophores, where we obtained an E_m of approximately −400 mV at pH 10.8 [19]. The discrepancy between this value, and that of Klimov et al. [48] probably lies in the redox mediators used in the respective titrations. They used 20 μM neutral red as a redox meditor, but it is not clear that such low levels of this relatively high midpoint potential dye (E_{m7} = −325 mV, see ref. 49) could mediate at −620 mV at pH 8.5. Indeed it is not clear how Klimov et al. achieved a stable ambient potential of −620 mV at pH 8.5, since this is appreciably below the value of the hydrogen electrode at this pH. Interestingly, Klimov et al. reported [48] that low levels of viologen dyes shifted the E_m of I/I$^{\cdot-}$ from their observed value of −620 mV to close to −400 mV, although they attributed this shift to an artefact induced by the viologens. The experiment of Fig. 5, performed in the absence of viologens, but still giving an E_m value of −400 mV, suggests that in fact the addition of viologens in the experiments of Klimov et al. [48] finally allowed mediation between I/I$^{\cdot-}$ and the measuring electrodes, rather than altering the equilibrium E_m value. However, although our experiments with *Rps. viridis* have been repeated several times with similar results, analogous but preliminary experiments with *Thiocapsa pfennigii* (which also possesses bacteriochlorophyll *b*) have so far failed to determine an E_m value for I/I$^{\cdot-}$ in this species, although I$^{\cdot-}$ is readily trapped by the illumination of appropriately poised samples at 200 K. Whether this reflects a true difference in E_m between the two species, or merely problems of equilibration in *T. pfennigii*, remains to be seen.

Interestingly the chemical reduction of I in *Rps. viridis* does not appear to generate the broad split EPR signal, although unpublished experiments show that the ability to generate this signal by illumination at 200 K attenuates with the same titration curve as the light induced triplet (see ref. 19). Instead the chemical reduction of I appears to generate only a narrow free radical EPR signal, although analysis of this is complicated because of the high levels of viologen redox mediators (which have stable free radicals in the reduced form) which are needed for efficient equilibration between I/I$^{\cdot-}$ and the measuring electrodes. Experiments to determine more clearly the spectroscopic properties of chemically reduced I$^{\cdot-}$ are currently in progress.

CONCLUSIONS

The major conclusion of this work is that there is no direct correlation between the two optical and the two EPR signals associated with I$^{\cdot-}$. Thus, there are optical changes which can be ascribed to the reduction of a bacteriopheophytin and a shift of the bacteriochlorophyll in LDAO-reaction centers, where the majority of the electrons of I$^{\cdot-}$ are in the form of the broad split EPR signal, but all the optical changes are also seen in SDS-reaction centers, where only the unsplit free radical is found.

If we ascribe all the optical changes of the bacteriochlorophylls to an apparent blue shift of their absorption maxima [10–18], we can then discuss all the EPR signals of I$^{\cdot-}$ as arising from PBh$^{\cdot-}$. The correlation between the presence of the *g* 1.82 signal prior to the trapping of I$^{\cdot-}$, with the presence of the broad split signal in I$^{\cdot-}$ strongly suggests that the broad signal represents the interaction of BPh$^{\cdot-}$ with Q$^{\cdot-}$Fe, while the shape of the broad split signal, and the extra "wings" that can be resolved in *Rps. viridis,* suggest that the interaction involves both exchange and dipolar coupling.

Interestingly it is apparently the iron of $Q^{\cdot -}$Fe which is essential for the production of the broad split signal; experiments to test this by substituting other metals into the reaction center are currently underway.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grants PCM 76 14209 and BMS 75-20252. D. M. T. acknowledges his National Research Service Award (NIH), J. P. T. a Guggenheim Memorial Fellowship, and P. L. D. his RCDA 1-K4-GM 70771.

REFERENCES

1 Straley, S. C., Parson, W. W., Mauzerall, D. C. and Clayton, R. K. (1973) Biochim. Biophys. Acta 305, 597–609

2 Parson, W. W., Clayton, R. K. and Cogdell, R. J. (1975) Biochim. Biophys. Acta 387, 265–278

3 Kaufmann, K. J., Dutton, P. L., Netzel, T. L., Leigh, J. S. and Rentzepis, P. M. (1975) Science 188, 1301–1304

4 Rockley, M. G., Windsor, M. W., Cogdell, R. J. and Parson, W. W. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2251–2255

5 Kaufmann, K. J., Petty, K. M., Dutton, P. L. and Rentzepis, P. M. (1976) Biochem. Biophys. Res. Commun. 70, 839–845

6 Dutton, P. L., Kaufmann, K. J., Chance, B. and Rentzepis, P. M. (1975) FEBS Lett. 60, 275–280

7 Fajer, J., Brune, D. C., Davis, M. S., Forman, A. and Spaulding, L. D. (1975) Proc. Natl. Acad. Sci. U.S. 72, 4956–4960

8 Dutton, P. L. and Prince, R. C. (1977) in The Photosynthetic Bacteria (Clayton, R. K. and Sistrom, W. R., eds.), Plenum Press, New York

9 Dutton, P. L. (1976) Photochem. Photobiol. 24, 655–657

10 Tiede, D. M., Prince, R. C., Reed, G. H. and Dutton, P. L. (1976) FEBS Lett. 65, 301–304

11 Tiede, D. M., Prince, R. C. and Dutton, P. L. (1976) Biochim. Biophys. Acta 449, 447–469

12 Dutton, P. L., Prince, R. C., Tiede, D. M., Petty, K. M., Kaufmann, K. J., Netzel, T. L. and Rentzepis, P. M. (1976) Brookhaven Symp. Biol. 28, 213–237

13 Netzel, T. L., Rentzepis, P. M., Tiede, D. M., Prince, R. C. and Dutton, P. L. (1977) Biochim. Biophys. Acta 460, 467–479

14 van Grondelle, R., Romijn, J. C. and Holmes, N. G. (1976) FEBS Lett. 72, 187–192

15 Shuvalov, V. A. and Klimov, V. V. (1976) Biochim. Biophys. Acta 440, 587–599

16 Trosper, T. L., Benson, D. L. and Thornber, J. P. (1976) Abstracts of the 4th Annual Meeting of the American Society for Photobiology, p. 63

17 Trosper, T. L., Benson, D. L. and Thornber, J. P. (1977) Biochim. Biophys. Acta 460, 318–330

18 Shuvalov, V. A., Krakhmaleva, I. N. and Klimov, V. V. (1976) Biochim. Biophys. Acta 449, 597–601

19 Prince, R. C., Leigh, J. S. and Dutton, P. L. (1976) Biochim. Biophys. Acta 440, 622–636

20 Dutton, P. L., Leigh, J. S. and Seibert, M. (1972) Biochem. Biophys. Res. Commun. 46, 406–413

21 Leigh, J. S. and Dutton, P. L. (1974) Biochim. Biophys. Acta 357, 67–77

22 Wraight, C. A., Leigh, J. S., Dutton, P. L. and Clayton, R. K. (1974) Biochim. Biophys. Acta 333, 401–403

24 Leigh, J. S. and Dutton, P. L. (1972) Biochem. Biophys. Res. Commun. 46, 414–421

25 Dutton, P. L., Leigh, J. S. and Reed, D. W. (1973) Biochim. Biophys. Acta 292, 654–664

26 Eimjhellen, K. E., Aasmundred, O. S. and Jensen, A. (1963) Biochem. Biophys. Res. Commun. 10, 232–236

27 Pucheu, N. L., Kerber, N. L. and Garcia, A. F. (1976) Arch. Microbiol. 109, 301–305

28 Siegelman, H. W. and Kycia, J. H. (1977) in Handbook of Physiological Methods (Cragle, J. and Hellebust, J. A., eds.), Cambridge University Press II

29 Thornber, J. P. and Trosper, T. (1977) Biochim. Biophys. Acta, in press

30 Loach, P. A. and Hall, R. L. (1973) Proc. Natl. Acad. Sci. U.S. 60, 786–790

31 Feher, G., Okamura, M. Y. and McElroy, J. D. (1972) Biochim. Biophys. Acta 267, 222–226

32 McElroy, J. D., Feher, G. and Mauzerall, D. C. (1972) Biochim. Biophys. Acta 267, 363–374

33 Thornber, J. P., Olson, J. M., Williams, D. M. and Clayton, M. L. (1969) Biochim. Biophys. Acta 172, 351–354

34 Dutton, P. L. and Wilson, D. F. (1974) Biochim. Biophys. Acta 346, 165–212

35 Lozier, R. H. and Butler, W. L. (1973) Biochim. Biophys. Acta 333, 460–464

36 Bolton, J. R. and Cost, K. (1973) Photochem. Photobiol. 18, 417–421

37 Leigh, J. S. and Dutton, P. L. (1972) Biochem. Biophys. Res. Commun. 46, 414–421

38 Dutton, P. L., Leigh, J. S. and Reed, D. W. (1973) Biochim. Biophys. Acta 292, 654–664

39 Feher, G., Isaacson, R. A., McElroy, J. D., Ackerson, L. C. and Okamura, M. Y. (1974) Biochim. Biophys. Acta 368, 135–139

40 Cogdell, R. J., Brune, D. C. and Clayton, R. K. (1974) FEBS Lett. 45, 344–347

41 Okamura, M. Y., Isaacson, R. A. and Feher, G. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3491–3495

42 Okamura, M. Y., Ackerson, L. C., Isaacson, R. A., Parson, W. W. and Feher, G. (1976) Biophys. J. 16, 233a

43 Garcia, A. F., Vernon, L. P., Ke, B. and Mollenhauer, H. H. (1968) Biochemistry 7, 326–332

44 Philipson, K. D. and Sauer, K. (1973) Biochemistry 12, 535–539

45 Fajer, J., Davis, M. S., Spaulding, L. D., Borg, D. C. and Forman, A. (1976) Brookhaven Symp. Biol. 28, 74–104

46 Vermeglio, A. and Clayton, R. K. (1976) Biochim. Biophys. Acta 449, 500–515

47 Prince, R. C. and Dutton, P. L. (1976) Arch. Biochem. Biophys. 172, 329–334

48 Klimov, V. V., Shuvalov, V. A., Krakhmaleva, I. N., Klevanik, A. V. and Krasnovskii, A. A. (1977) Biokhimija 42, 519–530

49 Clark, W. M. (1960) Oxidation-reduction potential of organic systems, Williams and Wilkins, Baltimore

50 Clayton, R. K. and Straley, S. C. (1972) Biophys. J. 12, 1221–1234

51 Shuvalov, V. A., Klimov, V. V., Krakhmaleva, I. N., Moskalenko, A. A. and Krasnovskii, A. A. (1976) Dokl. Biophys. 227, 62–65

52 Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 516–524