BBA 46316

CHARACTERIZATION OF PRIMARY REACTANTS IN BACTERIAL PHOTOSYNTHESIS

I. COMPARISON OF THE LIGHT-INDUCED EPR SIGNAL ($g = 2.0026$) WITH THAT OF A BACTERIOCHLOROPHYLL RADICAL

JAMES D. McELROY[a], GEORGE FEHER[a,*] AND DAVID C. MAUZERALL[b]

[a] *University of California, San Diego, La Jolla, Calif. 92037 (U.S.A.) and* [b] *The Rockefeller University, New York, N.Y. 10021 (U.S.A.)*

(Received December 16th, 1971)

SUMMARY

We show in this work that the light-induced free radical in *Rhodospirillum rubrum* arises from a radical of bacteriochlorophyll. The identification is based on the comparison of the EPR characteristics (g values and line widths) of the light-induced radical with those of a bacteriochlorophyll radical prepared *in vitro*. The g values are found to be identical ($g = 2.0026 \pm 0.0001$) within the experimental error. Comparison of the linewidth components of native and deuterated radicals at two microwave frequencies shows identity of several aspects of the electronic structure of the two radical species. The similarity of the line widths of the EPR signals in different bacterial species suggests that these conclusions can be generalized to the other photosynthetic bacteria.

INTRODUCTION

The first light-induced EPR signals in photosynthetic tissue were observed by Commoner *et al.*[1] in green plants and by Sogo *et al.*[2] in photosynthetic bacteria. Although these early measurements have been improved and extended, the chemical identification of the light-induced free radical from the basic features of the EPR signal has been difficult. In both the green plants and the photosynthetic bacteria, the g values of the EPR lines suggest that the signals arise from organic free radicals. The line shapes are Gaussian with line widths of about 7–10 G. No resolved hyperfine structure is observed, so the usual method of identifying radicals by the number, amplitude and splitting of the hyperfine lines is not applicable. In both cases, part of the line width is due to hyperfine interaction with hydrogen nuclei, as shown by the deuteration experiments of Kohl *et al.*[3].

Approaches to the identification of the light-induced free radical in photosynthetic bacteria have involved comparison of several properties of the light-

Abbreviations: EPR, electron paramagnetic resonance; ENDOR, electron-nuclear double resonance; NMR, nuclear magnetic resonance; P_{865}, specialized bacteriochlorophyll.

* To whom reprint requests should be addressed.

induced EPR signal with those of the photobleaching of the specialized bacteriochlorophyll molecule, P_{865}. These properties include action spectra[4,5], quantum yields[6,7], decay kinetics at room temperature[8,6] and at low temperature[9], and redox potentials[10]. On the basis of these comparisons[6–10], the light-induced radical has been identified as the oxidized P_{865} molecule which, in turn, has been associated with the primary photochemical act[11].

The approach to the identification of the light-induced radical used in this work is the comparison of its EPR characteristics with those of a suitable model radical. In view of the comparisons cited above, we have chosen bacteriochlorophyll as the model compound. Our goal is to demonstrate that the two radicals have identical electronic structure as measured by g values, hyperfine broadening and g anisotropy. Precisely measured g values are used to compare the two radicals. The unresolved proton hyperfine structure is investigated using the narrowing of the EPR signal of the light-induced radical[3] and the bacteriochlorophyll radical[9] upon deuteration. The anisotropy of the g value is studied using the broadening of the EPR signals with increasing magnetic field (microwave frequency).

The line width and g value of the light-induced free radical in photosynthetic bacteria are constant over the temperature range from 1–300 °K[12], suggesting that the radical is part of a rigid structure. In order to place the model radical into a rigid environment also, all EPR measurements were made using frozen samples at temperatures in the range of 77–110 °K.

A preliminary report of this work has been published[9].

MATERIALS AND METHODS

Bacteria

Cultures of *Rhodospirillum rubrum*, Strain 1, and *Rhodopseudomonas spheroides*, R-26, were grown in 1 l prescription flasks of modified Hutner medium[13]. The inoculated flasks were placed in a constant temperature water bath which was illuminated by rows of 40-W tungsten lamps (G. E. 40T8 showcase lamp) immersed in the water. The average light intensity at the surface of a culture flask was approximately 5 mW/cm^2 as measured by a YSI Model 65 radiometer over a wavelength range of 350–1000 nm. The temperature of the bath was held at 30 $\pm$ 2 °C. The cells were harvested by centrifugation after 3 days of growth. The yield was approximately 10 g (wet weight) of cells per l of culture.

R. rubrum was grown in deuterated medium[3] from a deuterated culture provided by Dr J. J. Katz. The degree of deuteration was greater than 99 % (J. J. Katz, personal communication). A preparation of chromatophores of *Chromatium*, Strain D, grown heterotrophically[14], was provided by Dr S. J. Kennel. Whole cells of *Rhodopseudomonas viridis* were provided by Dr J. P. Thornber[15].

Sample preparation

The harvested cells were washed to remove paramagnetic impurities and were suspended in buffer (either 0.01 M glycyl–glycine, pH 7.8 or 0.01 M Tris–HCl, pH 7.5) to a concentration corresponding to an absorbance of 50 (1-cm path) at the infrared peak of bacteriochlorophyll (*e.g.* at 865 nm for *R. spheroides*). The suspended cells

were transferred into quartz EPR tubes* and held in the dark for 2 min before freezing by immersion in liquid nitrogen. The samples were stored in liquid nitrogen and transferred to the EPR spectrometers without thawing. All operations were carried out aerobically in dim light. In some cases, the EPR signal was produced by addition of $K_3Fe(CN)_6$ up to 10^{-4} M final concentration. These samples were transferred immediately to EPR tubes and frozen.

Reaction centres of *R. spheroides*, R-26, were prepared as previously described[16]. EPR samples were prepared using concentrations corresponding to an absorbance of 30 (1-cm path) at 800 nm.

Bacteriochlorophyll radical

Bacteriochlorophyll was extracted from whole cells of *R. rubrum* using a mixture of 8 vol. acetone and 2 vol. distilled water. The acetone was removed by evaporation under a stream of dry nitrogen gas, and the precipitated bacteriochlorophyll was recovered from the water by centrifugation. The precipitate was redissolved in a mixture of 1 vol. ethanol and 4 vol. hexane and was applied to a packed column of commercial powdered sugar. The column was developed with hexane containing 0.75% isopropanol by volume**. The purified bacteriochlorophyll was extracted from the extruded sugar column with diethyl ether; the ether phase was then washed with water and evaporated to dryness. Purity of the bacteriochlorophyll was established by optical absorbance spectroscopy of the ether solution. Deuterated bacteriochlorophyll (degree of deuteration $> 99\%$) supplied by Dr J. J. Katz was purified by chromatography on powdered sugar columns as described above.

The bacteriochlorophyll radical was prepared by oxidizing bacteriochlorophyll in methanol with iodine. A 10^{-4} M solution of bacteriochlorophyll in a degassed mixture of 1 vol. methanol, 1 vol. glycerol was prepared, and 0.01 M I_2 in methanol, also degassed, was added in the closed, evacuated system to give a molar ratio of iodine to bacteriochlorophyll of approximately 10. The radical thus formed was stable at room temperature[17]. After the addition of iodine, the solution was transferred anaerobically to quartz EPR tubes and frozen immediately***. The deuterated bacteriochlorophyll radical was prepared in the same way except that the quartz capillary was sealed off after the sample was frozen; in this case the identical sample was used at both 9 and 35 GHz. All operations during the purification of bacteriochlorophyll and the preparation of the radicals were carried out in dim light.

EPR measurements

EPR measurements were carried out using a 9-GHz superheterodyne spectrometer of local design and a modified Varian V-4502 spectrometer operating at 9 and 35 GHz[12]. Low temperature measurements at 9 GHz employed the Varian V-4557 variable temperature apparatus; a conventional double dewar arrangement

* The inside diameter of the quartz tubes used at 9 GHz was 3 mm, and that of the tubes used at 35 GHz was 2 mm. Those tubes used for anaerobic preparations were fitted with ground joints.

** More recently we have used a mixture of 1 vol. diethyl ether, 8 vol. hexane to redissolve the precipitate and a mixture of one part ether, four parts hexane to develop the column.

*** No difference in EPR characteristics was observed between radicals prepared as described and radicals prepared aerobically and frozen immediately.

was used for measurements at 35 GHz. Care was taken to avoid distortion of EPR traces by time-constant effects, drifting base lines, or microwave passage effects. Magnetic field sweeps were controlled by a Varian Fieldial and were calibrated using a Varian F-8 NMR fluxmeter; magnetic fields were measured with an accuracy of $\pm$ 50 mG. Microwave frequencies were measured to one part in 10^6 using transfer oscillators and electronic counters. A signal-averaging computer (CAT 1000, Technical Instruments Corp.) was used to improve the signal-to-noise ratio of the light-induced EPR signals.

EXPERIMENTAL RESULTS AND ANALYSIS

g value measurements

The g value of an EPR line is given by the resonance condition

$$g = \frac{h\nu}{\beta \boldsymbol{H}_0} \tag{1}$$

where ν is the microwave frequency, $\boldsymbol{H}_0$ is the magnetic field at the center of the EPR line, h is Planck's constant, and β is the Bohr magneton. Information concerning the electronic structure of a free radical is contained in the departure of the measured g value from that of the free electron ($g_{\text{free electron}} = 2.00232$). These departures, termed g shifts, arise from an admixture of orbital angular momentum and are expected to be very small for aromatic free radicals[18] such as bacteriochlorophyll. Precise measurements of g values are therefore required to determine g shifts to the accuracy necessary for the characterization of these radicals. The errors ($\pm$0.0001) reported in Table I arise from the uncertainty in determining the center of the EPR line* (approximately 0.05 G).

TABLE I

g VALUES OF THE LIGHT-INDUCED FREE RADICAL OF *R. rubrum* AND THE OXIDIZED BACTERIOCHLOROPHYLL RADICAL

The g values are calculated from Eqn 1 of the text. The EPR signal of *R. rubrum* was induced by light from a quartz–iodine lamp filtered by a Corning C.S. 2-64 color filter. The light intensity was 30 mW/cm^2/s. The sample temperature was 110 °K for the 9-GHz measurements and approx. 85 °K for the 35-GHz measurements; the g values were independent of temperature over the range 1–300 °K. The bacteriochlorophyll radical was prepared as described in the text.

Species	*g (9 GHz)* ($\pm$ *0.0001*)	*g (35 GHz)* ($\pm$ *0.0001*)
R. rubrum		
native	2.0026	2.0026
deuterated	2.0026	2.0027 ($\pm$ 0.0002)
Oxidized bacteriochlorophyll		
native	2.0025	2.0025
deuterated	2.0025	2.0026

* It should be noted that, for a given signal-to-noise ratio, the uncertainty, $\delta \boldsymbol{H}$, in locating the center of the line, is given for a Gaussian line by

$$\delta \boldsymbol{H} \simeq \frac{\Delta \boldsymbol{H}}{\text{S/N}}$$

where $\Delta \boldsymbol{H}$ is the peak-to-peak derivative line width, and S/N is the signal-to-noise ratio of the peak of the derivative line.

The data of Table I show that the g values of the light-induced free radical of *R. rubrum* and the bacteriochlorophyll model radical are identical at both 9 and 35 GHz and for both the native and the deuterated species.

Line width measurements

The peaks of the derivative EPR lines were located by fitting a Gaussian curve to the experimental trace using the line width and amplitude as adjustable parameters. For all traces except the ones obtained from deuterated samples at 35 GHz, the experimental curves are well approximated by the Gaussian fit as can be seen for the 9-GHz data in the top part of Fig. 1. In the case of deuterated samples at 35 GHz, the two peaks were fitted separately because of slight (10 %) asymmetries in the peak heights. The errors quoted in Tables II and III are the root-mean square combination of the uncertainties in locating the peaks due to the finite signal-to-noise ratio.

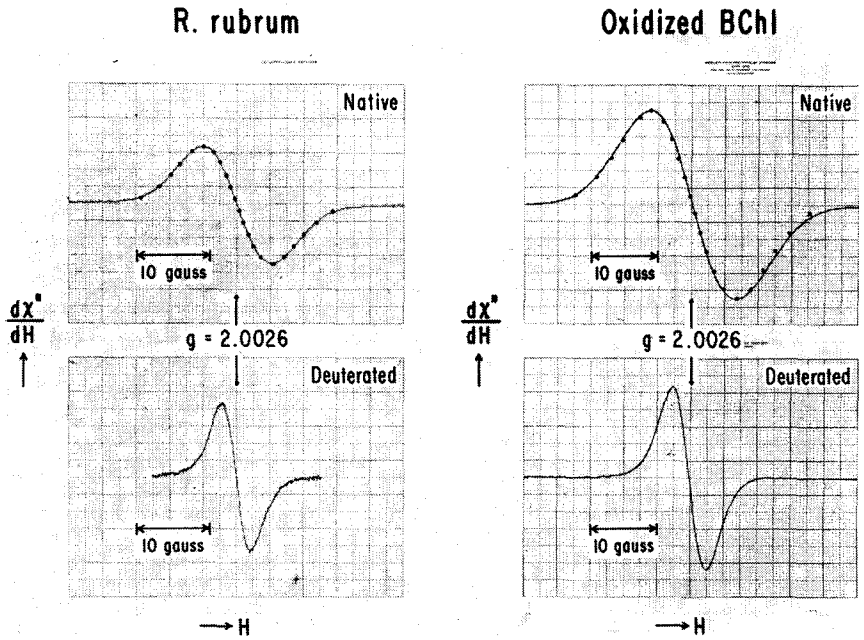

Fig. 1. EPR lines of the light-induced free radical in *R. rubrum* and the oxidized bacteriochlorophyll (BChl) radical at 9 GHz. The units of dX''/dH are arbitrary. The points on the lines of the native radicals represent a Gaussian fit. Experimental conditions were those of Table I.

The EPR lines of Fig. 1 demonstrate the narrowing upon deuteration which characterizes the light-induced free radical of *R. rubrum* and the bacteriochlorophyll model radical. The broadening of these lines with increasing microwave frequency is shown in Table II.

The line widths and broadening behavior observed for *R. rubrum* are similar to those observed for other purple photosynthetic bacteria (see Table III). Even *R. viridis*, which contains a different bacteriochlorophyll (bacteriochlorophyll *b*) and has a somewhat wider line, shows a broadening at the higher frequency. The EPR line of whole cells of *R. spheroides*, R-26, is slightly narrower than that of reaction

TABLE II

LINE WIDTHS OF THE LIGHT-INDUCED FREE RADICAL OF *R. rubrum* AND THE OXIDIZED BACTERIOCHLOROPHYLL RADICAL

The peak-to-peak widths of the derivative EPR lines are listed. The errors quoted are the root-mean square combinations of uncertainties in locating the peaks. Experimental conditions are those of Table I.

Species	ΔH (9 GHz) (G)	ΔH (35 GHz) (G)
R. rubrum *		
normal	9.4 ± 0.2	10.3 ± 0.2
deuterated	3.9 ± 0.2	5.9 ± 0.3**
Bacteriochlorophyll		
normal	13.0 ± 0.2	14.2 ± 0.2
deuterated	5.0 ± 0.2	7.1 ± 0.2

* The linewidths obtained by Kohl *et al.*[3] for *R. rubrum* at 9 GHz were $\Delta H_{native} = 9.1 \pm 0.5$ G and $\Delta H_{deuterated} = 4.0 \pm 0.5$ G.

** The linewidth of deuterated *R. rubrum* was measured at 1.4 °K. The width of the light-induced EPR line does not vary with temperature over the range of 1–300 °K.

TABLE III

LINE WIDTHS OF SOME PHOTOSYNTHETIC BACTERIA

The peak-to-peak widths of the derivative EPR lines are listed. The errors quoted are the root-mean square combination of the uncertainties in locating the peaks. Experimental conditions are those of Table I.

Species	*Preparation*	ΔH_{PTP} (9 GHz) (G)	ΔH_{PTP} (35 GHz) (G)
R. rubrum Strain 1	Whole cells, light-induced	9.4 ± 0.2	10.3 ± 0.2
R. rubrum deuterated	Whole cells, light-induced	3.9 ± 0.2	5.9 ± 0.3
R. spheroides, R-26	Whole cells, light-induced	9.3 ± 0.2	10.2 ± 0.2
R. spheroides, R-26	Reaction centers, light-induced	9.8 ± 0.2	10.7 ± 0.2
R. spheroides, R-26	Reaction centers *plus* $K_3Fe(CN)_6$	9.8 ± 0.2	10.8 ± 0.2
Chromatium Strain D	Chromatophore, light-induced	10.1 ± 0.3	10.7 ± 0.3
R. viridis	Whole cells, light-induced	12.2 ± 0.5	12 ± 1
R. viridis	Whole cells *plus* $K_3Fe(CN)_6$	12.0 ± 0.2	12.6 ± 0.2

centers prepared from the cells. This difference may be caused by small changes in the bacteriochlorophyll–protein complex which occur when the reaction centers are extracted with detergents.

Line width decomposition

The goal of this investigation is to compare the EPR characteristics of the light-induced free radical with the bacteriochlorophyll model radical. The total peak-to-peak line width provides only one point of comparison. However, since several independent broadening mechanisms contribute to the observed width, a decomposition of the line widths into components will provide a more meaningful comparison. In this section, we shall discuss the broadening mechanisms and how the decomposition is accomplished.

Broadening due to unresolved hyperfine interactions: If the hyperfine interaction of the unpaired spin with its surrounding nuclei is small, the hyperfine lines will not be resolved, but will contribute to the width of the EPR signal. The resulting line is said to be inhomogeneously broadened[19]. The two characteristic features of such a line are that its shape is Gaussian and that it shows a characteristic saturation behavior with increasing microwave power[20]. Fig. 1 shows the Gaussian shape for the two radicals. Measurements of the microwave power saturation behavior of the light-induced free radical[12,21,22] and the bacteriochlorophyll radical[12] at 9 GHz provides additional proof that both species have inhomogeneously broadened EPR lines.

As we have already mentioned, it has been established that hyperfine interaction with hydrogen nuclei contributes to the width of both the light-induced radical and the bacteriochlorophyll radical. (The experimental observation in both cases was that the EPR line of the deuterated species was narrower than that of the corresponding native species of the particular radical.) The dependence of the hyperfine component, $\Delta \boldsymbol{H}_i$, on the nuclear spin I_i and the nuclear gyromagnetic ratio γ_I is given by[20]

$$\Delta \boldsymbol{H}_i \propto \gamma_i \sqrt{I_i(I_i + 1)} \tag{2}$$

Substituting the values for protons and deuterons, respectively, into Eqn 2, we find that, if the line widths were due entirely to hyperfine interactions with hydrogen nuclei, the lines should narrow by a factor of 3.99 upon deuteration. From Table II we see that this is not the case, indicating the presence of other broadening mechanisms. (At 9.2 GHz, the ratio of native to deuterated line width is 2.4 for *R. rubrum* and 2.6 for bacteriochlorophyll.) An obvious candidate for the additional broadening is hyperfine interaction with nuclei other than hydrogen. Let us denote this contribution by $\Delta \boldsymbol{H}_n$ since, in our case, the four pyrrole nitrogens of the bacteriochlorophyll molecule are most likely to contribute to hyperfine broadening*.

The line width due to hyperfine interactions is expected to be independent of the external magnetic field (*i.e.* microwave frequency)[19]. However, we find experimentally that the lines broaden with increasing magnetic field (see Table II). We have to consider, therefore, an additional, different line broadening mechanism.

Broadening due to g anisotropy: If the g value of the EPR line depends on the orientation of the radical molecule with respect to the external magnetic field, a random orientation of the radicals will result in an EPR line containing components resonating at different magnetic fields. Thus, not only will the line be broadened by the g anisotropy, but the line width will increase with increasing magnetic field (microwave frequency). The problem of determining the relative contributions of this effect and of hyperfine interaction to the observed line widths is a difficult one because of the complex nature of broadening by g anisotropy. Several authors have published numerical calculations of the line shapes of anisotropic radicals[24,25].

* Fajer *et al.*[23] suggest that the electron density on the pyrrole nitrogens in π-cation radicals of metalloporphyrins depends critically on the electronic ground state configuration. For the zinc tetraphenyl porphyrin radical, they found a splitting constant A_n of 1.58 G, whereas for magnesium octaethylporphyrin $A_n < 0.3$ G.

However, since we are interested in comparisons rather than absolute values of g anisotropies, we will use a simplified approach to the problem. It suffices to note that for small anisotropies, the resulting line width component, ΔH_g, will be proportional to the g anisotropy, Δg, and the external magnetic field, H_0 (see Eqn 1), *i.e.*

$$\Delta H_g \simeq \frac{\Delta g}{g} H_0 \tag{3}$$

Thus, by working at two magnetic fields (*i.e.* two microwave frequencies) we can unravel the relative effects of g anisotropy and hyperfine interaction, the latter being independent of magnetic field. For example, the contribution of g anisotropy to the line width at 35 GHz is 3.8 times greater than the corresponding contribution at 9.2 GHz.

Evaluation of the individual components contributing to the line width: As we have mentioned above, the exact determination of the contribution of the different broadening mechanisms to the observed line width is difficult because of the complex line shape produced by the g anisotropy. However, irrespective of detailed line shape arguments, one can show that for independent line broadening mechanisms the second moment of the observed EPR line, $\langle \Delta H^2_{ob} \rangle$, is the sum of the second moments of the individual components, $\langle \Delta H^2_i \rangle$. We shall use this relation in the analysis which follows. However, rather than determining the second moments by double numerical integration of the observed derivative spectra, we shall use the fact that the second moment is proportional to the peak-to-peak derivative line width for the Gaussian line shapes observed*. Assuming that the proportionality factor for Gaussian lines is valid for all observed line widths and all line width components, we can write the expression for the second moment in terms of the experimentally determined peak-to-peak derivative line widths.

$$\Delta H^2_{H,D}(\nu) = \Delta H^2_{h,d} + \Delta H^2_n + \Delta H^2_g(\nu) \tag{4}$$

where $\Delta H_H(\nu)$ and $\Delta H_D(\nu)$ are the observed peak-to-peak line widths for the native and deuterated species at microwave frequency ν (9.2 and 35 GHz), and ΔH_h, ΔH_d, and ΔH_n are the peak-to-peak line width components due to hyperfine interactions with protons, deuterons, and nitrogen nuclei, respectively. $\Delta H_g(\nu)$ is the g-anisotropy component at microwave frequency.

The above relation represents four equations for a native and deuterated radical species at the two microwave frequencies. Together with the subsidiary relations,

$$\Delta H_h/\Delta H_d = 3.99; \qquad \Delta H_g(35)/\Delta H_g(9.2) = \frac{35}{9.2} = 3.8 \tag{5}$$

we have six equations to determine five quantities (ΔH_h, ΔH_d, ΔH_n, ΔH_g (35) and ΔH_g (9.2)). The problem is therefore over-determined. This enables us to make a judicious choice in picking the set of equations that will minimize the errors introduced by the finite signal-to-noise ratio and by the assumption made in writing Eqn 4 (*i.e.* that the lines are strictly Gaussian).

* As mentioned above, the line shapes obtained from deuterated samples at 35 GHz deviate slightlp from Gaussian. The errors introduced bp this deviation in the determination of ΔH_h, ΔH_d, ΔH_n have been minimized, as explained in the text.

The component due to g anisotropy, $\Delta H_g(\nu)$, is small and has a more pronounced contribution in the narrower lines. Therefore, we determine $\Delta H_g(35)$ by subtracting the equations of Relation 4 which involve the line widths of the deuterated radical at 35 and 9.2 GHz. We obtain

$$\Delta H_g^2(35) = \frac{\Delta H_D^2(35) - \Delta H_D^2(9.2)}{1 - \Delta H_g^2(9.2)/\Delta H_g^2(35)} = 1.074[\Delta H_D^2(35) - \Delta H_D^2(9.2)] \quad (6)$$

The proton component, ΔH_h, is best obtained from the 9.2-GHz data. At this lower frequency, the anisotropy does not contribute significantly to the line width. The approximate manner in which we have treated ΔH_g therefore causes a negligible error in the determination of ΔH_h. From Eqn 4, we obtain

$$\Delta H_h^2\ (9.2) = \frac{\Delta H_H^2(9.2) - \Delta H_D^2(9.2)}{1 - \Delta H_d^2\ /\Delta H_h^2} = 1.067[\Delta H_H^2(9.2) - \Delta H^2(9.2)] \quad (7)$$

The line width component due to the nitrogen hyperfine interaction is also obtained from the 9.2-GHz data (the argument is the same as used for ΔH_h). The effect of the nitrogen component is more pronounced in the deuterated samples which have the narrower lines. Using the line widths of the deuterated radicals, we obtain from Eqn 4,

$$\Delta H_n^2 = \Delta H_D^2(9.2) - \Delta H_d^2 - \Delta H_g^2(9.2) \quad (8)$$

The relation between ΔH_n and the hyperfine splitting constant, A_n, which, in principle, can be obtained from an electron-nuclear double resonance (ENDOR) experiment[26], is given for a Gaussian line by

$$\Delta H_n = \frac{2}{\sqrt{3}}\sqrt{NI(I+1)}\,A_n \quad (9)$$

where N is the number of equivalent nuclei with nuclear spin I[19]. (The expression is exact only in the limit of large N. For four equivalent nuclei with $I = 1$, it is accurate to about 10 %.)

TABLE IV

COMPARISON OF THE LINE WIDTH COMPONENTS OF THE LIGHT-INDUCED RADICAL AND THE BACTERIOCHLOROPHYLL RADICAL

The line width of a native radical is assumed to have three components: ΔH_h, the component due to proton hyperfine interactions; ΔH_n, the component the to hyperfine interaction with the nuclei of the pyrrole nitrogens, and $\Delta H_g(\nu)$, the frequency-dependent component due to anisotropy of the electronic g value. The decomposition analysis is described in the text, Eqns 4–8.

Species	ΔH_h (G)	ΔH_n (G)	ΔH_g (9 GHz) (G)	$\Delta H_h/\Delta H_n$	A_n* (G)
*R. rubrum***	8.8 ± 0.3	3.0 ± 0.3	1.2 ± 0.1	3.0 ± 0.3	0.91 ± 0.09
Bacteriochlorophyll	12.4 ± 0.3	3.7 ± 0.3	1.4 ± 0.1	3.4 ± 0.3	1.13 ± 0.09

* Computed using Eqn 9, assuming four equivalent nuclei of spin 1.

** The values obtained by Kohl *et al.*[3] for *R. rubrum* at 9 GHz were $\Delta H_h = 8.8 \pm 0.6$ G, $\Delta H_x = 3.4 \pm 0.7$ G, where ΔH_x was due to an unspecified broadening mechanism.

The results of the above analysis for both radical species are summarized in Table IV. Two striking features emerge: the ratio $\Delta H_h/\Delta H_n$ is the same within experimental error for both radical species, and the value of ΔH_h of *R. rubrum* is 40% smaller than that of bacteriochlorophyll. The significance of these findings is discussed in the following section.

DISCUSSION

In this section we wish to summarize and discuss the evidence presented in this work showing that the narrow, light-induced EPR signal of photosynthetic bacteria is associated with a bacteriochlorophyll radical.

The first piece of evidence is provided by the identity of the g values of the light induced free radical and the bacteriochlorophyll free radical. Although the g value is not an extremely sensitive indicator of the nature of a radical molecule, the observed value of 2.0026 ± 0.0001 is characteristic of aromatic π radicals[18] including, besides oxidized bacteriochlorophyll, also oxidized chlorophyll[27] and some metalloporphyrin radicals[17,23]. Other classes of organic radicals have g values which are characteristically different; for example, the g value of the reduced semiquinone radical of coenzyme Q_{10} is 2.0047[28], while those of a series of flavin radicals fall in the range 2.0030–2.0034[29].

The second and stronger piece of evidence is provided by the comparison of the line broadening mechanisms in the two radical species (see Table IV). We start out by comparing the values of $\Delta H_h/\Delta H_n$ for the two radicals. This quantity is a measure of the relative distribution of the unpaired spin over the nuclear sites and should, therefore, be a sensitive measure of the electronic structure of the molecule. As can be seen from Table IV, the ratio is the same within experimental error for both radical species. Similarly ΔH_g, which reflects the anisotropy of the electronic structure is, within experimental error, the same for both radicals.

We turn now to a comparison of the absolute values of ΔH_h and ΔH_n obtained for the two radicals. These quantities are proportional to the probabilities of finding the unpaired spin at the respective nuclei. From Table IV we find that

$$\frac{\Delta H_h(\text{bacteriochlorophyll})}{\Delta H_h(R.\,rubrum)} = 1.41 \pm 0.05; \quad \frac{\Delta H_n(\text{bacteriochlorophyll})}{\Delta H_n(R.\,rubrum)} = 1.24 \pm 0.16 \quad (10)$$

Can we reconcile these sizable differences with the identity of the light-induced radical and the bacteriochlorophyll radical which is implied by the identity of the g values and line broadening mechanisms? We have argued before that such a difference may arise from the different environments *in situ* and *in vitro*[9]. An alternate explanation of the differing line width components has been advanced recently by Norris, *et al.*[30]. These authors postulate that the unpaired spin in the light-induced free radical is shared equally between a pair of bacteriochlorophyll molecules, resulting in hyperfine components which are smaller than those of an isolated bacteriochlorophyll radical by a factor $\sqrt{2}$. The fact that the ratio of our experimentally determined line width components due to protons is equal to $\sqrt{2}$, and that due to nitrogens comes close to $\sqrt{2}$, leads us to favor this hypothesis at present.

It is worth noting that the hypothesis could be tested if a direct measurement of the hyperfine splitting constant, A_n, were available; Eqn 9 would yield then the number of nuclei contributing to ΔH_n. The ENDOR technique[26] provides a direct measure of A; we are currently applying this technique to the light-induced free radical and the bacteriochlorophyll radical.

So far, we have not touched on the question of whether the light-induced free radical is oxidized or reduced. Since our comparison involves oxidized bacteriochlorophyll, one is inclined to conclude that the light-induced radical is also oxidized. However, the cation and anion radicals of many aromatic compounds have remarkably similar EPR characteristics[18]. Therefore, in the absence of data on the reduced bacteriochlorophyll radical, we are unable to decide this question on the strength of the evidence presented here. Evidence obtained from other experiments[9-11] (J. D. McElroy, G. Feher and D. C. Mauzerall, Part II of this work, to be published), however, points to the fact that we are indeed dealing with the oxidized species (see also Note added in proof).

Before we conclude the discussion of our results, it seems appropriate to consider the arguments against the identification of the light-induced EPR signal with a bacteriochlorophyll radical which were presented by Kohl *et al.*[3] in a similar study. One argument was based on the assumption that a protonated or deuterated substrate (succinate) in the biosynthesis of bacteriochlorophyll in *R. rubrum* contributed the same number of protons or deuterons to the bacteriochlorophyll molecule irrespective of the isotopic composition of the medium. This assumption was shown later to be invalid by a set of "isotope mirror" experiments[31,32]. Another argument was based on the observation that no narrowing of the EPR line was seen in *Chlorella* when ^{15}N was substituted for ^{14}N in the growth medium. The expected narrowing of the line width component, ΔH_n, upon ^{15}N substitution is obtained from Eqn 2:

$$\frac{\Delta H_n(^{15}N)}{\Delta H_n(^{14}N)} = 0.87.$$

Under the most favorable conditions, *i.e.* in deuterated *R. rubrum*, our observed line width would be reduced from 3.9 to 3.6 G upon ^{15}N substitution. This narrowing is less than the line width errors reported by Kohl *et al.*[3].

We conclude, therefore, that the light-induced free radical in *R. rubrum* is a bacteriochlorophyll radical, noting the possibility that the unpaired spin is shared between two bacteriochlorophyll molecules. In view of the similarity of the width of the light-induced EPR signal in different bacterial species (see Table III), it is reasonable to extend this conclusion to the other photosynthetic bacteria. Furthermore, g value and line width measurements of the EPR signal of System I in algae and green plants made by other workers[27,30,33] suggest that the light-induced free radical of System I is a chlorophyll radical.

NOTE ADDED IN PROOF (Received April 28th, 1972)

The EPR spectrum of the bacteriochlorophyll anion radical $BChl^-$ has recently been observed by R. H. Felton, D. Dolphin, J. Fajer and A. Forman (personal communication). The EPR line has a g value of 2.0026 ± 0.0002 and shows a partially

resolved structure at room temperature. No low temperature data are available at present to compare its spectrum with that observed from the immobilized $BChl^+$ radical.

ACKNOWLEDGMENTS

We would like to thank Dr J. J. Katz of the Argonne National Laboratory for his gifts of a culture of deuterated bacteria and of a sample of deuterated bacteriochlorophyll. We are indebted to Dr M. D. Kamen and his staff at the University of California, San Diego for the use of some of their facilities and for many stimulating discussions. Helpful discussions with Dr H. Shore and the technical assistance of Mr. R. A. Isaacson are gratefully acknowledged. This work was supported by National Institutes of Health grant GM13191 and by the National Science Foundation. J. D. M. was supported in part by a Special Fellowship (GM 44384) from the National Institute of General Medical Sciences. D. C. M. acknowledges the support of a Guggenheim Fellowship. This work was carried out in partial fulfillment of the requirements for the doctorate of J. D. M.

REFERENCES

1 B. Commoner, J. J. Heise and J. Townsend, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 710.
2 P. Sogo, M. Jost and M. Calvin, *Radiat. Res. Suppl.*, 1 (1959) 511.
3 D. H. Kohl, J. Townsend, G. Commoner, H. L. Crespi, R. C. Dougherty and J. J. Katz, *Nature*, 206 (1965) 1105.
4 R. H. Ruby, *UCRL*, 16492 (1966) 52.
5 R. K. Clayton and W. R. Sistrom, *Photochem. Photobiol.*, 5 (1966) 661.
6 J. R. Bolton, R. K. Clayton and D. W. Reed, *Photochem. Photobiol.*, 9 (1969) 209.
7 P. A. Loach and K. Walsh, *Biochemistry*, 8 (1969) 1908.
8 P. A. Loach and D. L. Sekura, *Biochemistry*, 7 (1968) 2642.
9 J. D. McElroy, G. Feher and D. C. Mauzerall, *Biochim. Biophys. Acta*, 172 (1969) 180.
10 P. A. Loach, G. M. Androes, A. F. Maksim and M. Calvin, *Photochem. Photobiol.*, 2 (1963) 443.
11 W. W. Parson, *Biochim. Biophys. Acta*, 153 (1968) 248.
12 J. D. McElroy, Dissertation, University of California, San Diego, 1970.
13 H. DeKlerk, R. G. Bartsch and M. D. Kamen, *Biochim. Biophys. Acta*, 97 (1965) 275.
14 M. A. Cusanovich and M. D. Kamen, *Biochim. Biophys. Acta*, 153 (1968) 376.
15 J. P. Thornber, *Biochim. Biophys. Acta*, 172 (1969) 351.
16 G. Feher, *Photochem. Photobiol.*, 14 (1971) 373.
17 J. H. Fuhrhop and D. C. Mauzerall, *J. Am. Chem. Soc.*, 91 (1969) 4174.
18 B. G. Segal, M. Kaplan and G. K. Fraenkel, *J. Chem. Phys.*, 43 (1965) 4191.
19 A. F. Kip, C. Kittel, R. A. Levy and A. M. Portis, *Phys. Rev.*, 91 (1953) 1066.
20 A. M. Portis, *Phys. Rev.*, 91 (1953) 1071.
21 G. M. Androes, M. F. Singleton and M. Calvin, *Biochim. Biophys. Acta*, 66 (1963) 180.
22 H. Schleyer, *Biochim. Biophys. Acta*, 153 (1968) 427.
23 J. Fajer, D. C. Borg, A. Forman, D. Dolphin and R. H. Felton, *J. Am. Chem. Soc.*, 92 (1970) 3451.
24 C. P. Poole, Jr, *Electron Spin Resonance*, Interscience Publishers, New York, 1967, p. 835.
25 M. M. Malley, *J. Mol. Spectrosc.*, 17 (1965) 210.
26 G. Feher, *Phys. Rev.*, 103 (1956) 834.
27 D. C. Borg, J. Fajer, R. H. Felton and D. Dolphin, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 813.
28 M. S. Blois, Jr and J. E. Maling, *Biochem. Biophys. Res. Commun.*, 3 (1960) 132.
29 L. E. G. Eriksson and Anders Ehrenberg, *Acta Chem. Scand.*, 18 (1964) 1437.
30 J. R. Norris, R. A. Uphaus, H. L. Crespi and J. J. Katz, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 625.
31 R. C. Dougherty, H. L. Crespi, H. H. Strain and J. J. Katz, *J. Am. Chem. Soc.*, 88 (1966) 2854.
32 J. J. Katz, R. C. Dougherty, H. L. Crespi and H. H. Strain, *J. Am. Chem. Soc.*, 88 (1966) 2856.
33 E. C. Weaver, *Annu. Rev. Plant Physiol.*, 19 (1968) 283.