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An Electron–Nuclear Double Resonance (Endor) Study of the Special Pair Model for Photo-Reactive Chlorophyll in Photosynthesis

(hyperfine coupling constants/photo electron spin resonance/photosynthetic bacteria/selective deuteration)

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ABSTRACT **A comparison of hyperfine coupling constants obtained by electron–nuclear double resonance spectroscopy of *in vitro* monomer chlorophyll and bacteriochlorophyll free radicals with those of the photo-esr (electron spin resonance) signal associated with light conversion in photosynthesis provides convincing support for the special pair model for the *in vivo* photo-reaction center.**

The discovery that the primary event in photosynthesis is associated with an esr signal was made by Commoner *et al.* (1) in 1956. This photo-signal has a Gaussian line shape, a g-value of 2.0025, and a linewidth ΔH_{pp} of about 10 G. Esr investigations with ^{1}H-, ^{2}H-, ^{13}C-, and ^{25}Mg-organisms and their corresponding chlorophylls (2–10) have demonstrated the π-nature of the *in vivo* photo-esr signal. Quantitative and kinetic correlations of the esr and optical spectra have established that the photo-esr signal originates (as originally suspected by Commoner *et al.*) in a chlorophyll (or bacteriochlorophyll) doublet state free radical (Chl a^+ or Bchl$^+$) (11–16).

However, certain anomalous features of the *in vivo* chlorophyll free radical have been ignored until recently (8). In both green plants and photosynthetic bacteria, the *in vivo* esr signal is no less than 40% narrower than the signal from monomer Chl a^+ *in vitro*. Photo-reactive chlorophyll in green plants absorbs light maximally near 700 nm (17) and in bacteria near 865 nm, whereas monomer Chl *a* and Bchl absorb at 663 nm and 770 nm, respectively. These discrepancies can now be resolved by comparison with the properties of *in vitro* chlorophyll species that have been characterized by infrared, electronic transition, and magnetic resonance spectroscopy (18).

Studies over the past decade have established chlorophyll (Structure I) as a compound with an unusual combination of electron donor and acceptor properties (19). One of the most important of the species generated by donor–acceptor interactions is a chlorophyll–water adduct, $(\text{Chl } a \cdot H_2O)_n$, which is photo-active in red light by the esr criterion. The $(\text{Chl } a \cdot H_2O)_n$ adduct has a visible absorption spectrum that is highly red-shifted (λ_{max} = 740 nm), and has a remarkably narrow photo-reversible esr signal, with $\Delta H_{pp} \simeq 1$ G (20).

Abbreviations: Chl, the chlorophylls generically; Chl *a* and *b*, chlorophylls *a* and *b;* Bchl, bacteriochlorophyll *a;* Mpchl *a*, methyl pyrochlorophyllide *a;* Chl a^+, Mpchl a^+, Bchl$^+$, schematic representation of monomer free radicals; esr, electron spin resonance; endor, electron–nuclear double resonance; hf, hyperfine; ΔH, linewidth.

The unusually narrow esr line recorded from $(\text{Chl} \cdot H_2O)_n^+$ can be rationalized by a process of spin delocalization over many chlorophyll molecules (8, 20, 21). Simple, but rigorous, considerations show that delocalization of an unpaired spin over N chlorophyll molecules should narrow the (Gaussian) signal characteristic of monomer Chl a^+ or Bchl$^+$ by $1/\sqrt{N}$. Spin delocalization can be used equally well to account for the narrowing of the *in vivo* signal. A relationship $\Delta H_{in\ vitro}/\Delta H_{in\ vivo} \simeq \sqrt{2}$, in fact, accounts very well for the *in vivo* linewidth, and is the basis for the hypothesis that a photo-active chlorophyll in the photo-reaction center consists of a special pair of chlorophyll molecules (8). As the Chl *a*– and Bchl–water adducts are the only photo-active species so far prepared *in vitro*, it is reasonable to use them as a model or paradigm of *in vivo* photo-active Chl.

The special pair model for photo-active chlorophyll predicts that the esr line shape will be narrowed by a factor $1/\sqrt{2}$ relative to monomer Chl a^+, and that each and every electron-nuclear hyperfine (hf) coupling constant in a special pair will be reduced by a factor of 1/2 relative to the monomer coupling constants. A comparison of hf splittings measured in both *in vivo* and *in vitro* chlorophyll free radicals is thus a considerably more rigorous test of the special pair proposal. Because chlorophyll free radical esr signals, especially *in vivo*, do not show hf structures that permit extraction of hf coupling constants, we have had resort to electron–nuclear double resonance (endor) spectroscopy, a high resolution extension of esr first discovered by G. Feher (22), which in combination with organisms and chlorophylls of unnatural isotopic composition makes assignment of the endor spectrum and hf coupling constants possible.

MATERIALS AND METHODS

Instrumentation. Endor spectra were recorded at 10°K and 108°K on a modified Varian E-700 spectrometer.

Production of Chlorophyll Free Radicals. In vitro free radicals were usually generated in 10^{-4}–10^{-3} M Chl solutions in thoroughly degassed $C^2H_2Cl_2$–$C^2H_3O^2H$ by chemical oxidation with minimal amounts of iodine (23), ferric chloride, or zinc tetraphenylporphyrin free radical (24).

In vivo free radicals for endor were generated in packed whole cells, chromatophores, chloroplasts, and reaction center preparations by careful titration with $K_3Fe(CN)_6$. The esr and endor signals produced by this chemical method are indistinguishable from the photo-induced signal in bacteria (16) and green algae and thus are believed to have the same origin.

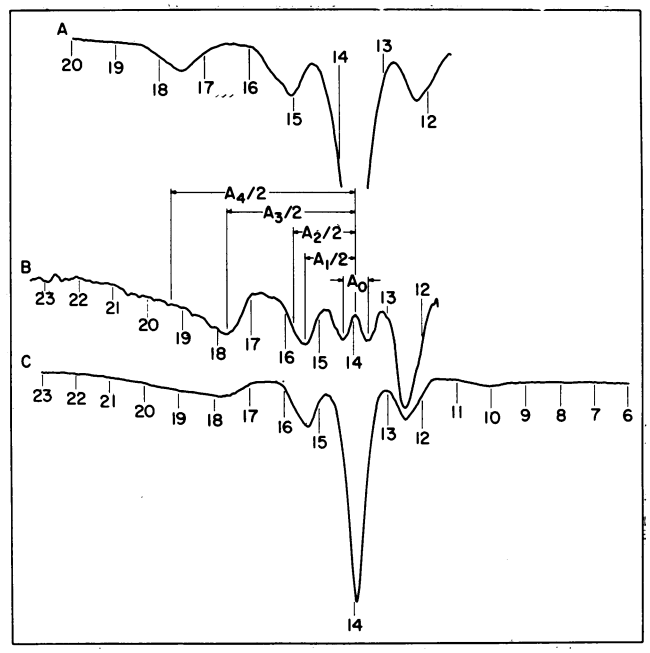


FIG. 1. Typical Chl endor spectra at 110°K. The hyperfine coupling constants are labeled from A_0 to A_4 as indicated in B and the frequency scale is in units of MHz. (A) Chl *a* in CH_2Cl_2–CH_3OH (4:1) oxidized by zinc tetraphenylporphyrin perchlorate; (B) Mpchl *a* in $C^2H_2Cl_2$–$C^2H_3O^2H$ (4:1) oxidized by I_2; (c) Mpchl *a* in which the 5a-methyl protons are substituted by deuterium. Solvent system as in (A). Note the disappearance of the A_3 coupling near 18 MHz.

RESULTS

In Vitro Chl a^+ Endor Assignments. Two strategies have been used to assign the *in vitro* Chl a^+ and Bchl$^+$ hf splittings. The side chains or the ring structure can be chemically altered, and the effects of chemical modification on the endor spectrum can then be ascertained. Or there is the introduction of ^2H in known sites in the chlorophyll molecule. Replacement of ^1H by ^2H in a particular site causes the endor peak contributed by that particular ^1H to vanish, thus making the assignment possible. The problem of isotopic labeling of the highly reactive chlorophylls is greatly simplified by the use of a suitable derivative, which must be more stable than Chl *a*, must have a relatively straightforward and well-established chemistry, and have a spin distribution in its free radical that is the same as in Chl a^+, i.e., it must have an endor spectrum that is virtually identical to that of Chl a^+. Methyl pyrochlorophyllide *a* (Mpchl *a*) (Structure II) is the compound of choice, primarily because magnesium can readily be reinserted after adjustment of the isotopic composition. A comparison of the endor spectra (Figs. 1A and B) shows that Chl a^+ and Mpchl a^+ have essentially identical endor spectra, satisfying the spin distribution criterion. Replacement of the phytyl group of Chl *a* with a methyl group, replacement of the carbomethoxy group at position 10 by ^1H, and reduction of the vinyl group at position 2 have no noticeable effect on the endor spectrum, indicative of very little spin density and very small coupling constants at these sites. Reduction of the 9-keto group of Mpchl *a* increases the 5a-methyl hf interaction to a significant extent, which indicates the proper assignment for the 5a-CH_3 group (see below). Conversion of ring V into a six-membered ring lactone decreases the 5a coupling constants, and in chlorin e_6 trimethylester, which no longer has an intact ring V, the 1a- and 5a-CH_3 groups, instead of differing almost by a factor of 2, have nearly the same hf coupling constants.

TABLE 1. *Comparison of in vitro and in vivo endor data*

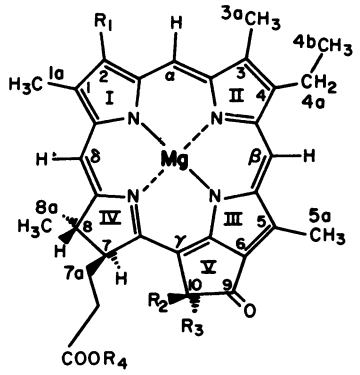
Protons[b]	Hyperfine coupling constants (MHz) Bchl$^+$	Chl a^+	Aggregation number[a] *R. rubrum*[c]	*S. lividus*[d,i]
(α, β, δ, 10)	1.4[e]	0.67[f]	1.7	—
1a	5.32	—	2.4	—
(1a, 3a, 4a)	—	3.19[g]	—	1.9
		3.72[g]		2.2
5a	9.8	7.45	2.1	2.0
7, 8	14.0[h]	11.8	2.0	2.2
			Av. 2.0	2.1

[a] Defined as the ratio of hf coupling constants *in vitro*/hf coupling constants *in vivo*.
[b] Proton numbering from Structure I. Parentheses indicate the hf coupling constant arise from all of the indicated protons.
[c] Endor recorded at 15°K.
[d] Endor recorded at 100°K.
[e] Includes contributions from 7a, 8a, 3a, and 4a protons.
[f] Includes contributions from 7a and 8a protons.
[g] These peaks are resolved but are not assigned.
[h] Includes contributions from 3 and 4 protons.
[i] Endor from *C. vulgaris* and *S. lividus* are indistinguishable.

The way in which ^2H substitution facilitates hyperfine coupling assignments is shown in Fig. 1C. Deuteration of the 5a-CH_3 groups of Mpchl *a* causes the disappearance of a prominent resonance peak (Fig. 1C), which confirms the assignment of the prominent endor line at 17.5 MHz to the unpaired electron interaction with the protons of the 5a-methyl group. More than a dozen chemical derivatives and isotopically substituted chlorophylls were prepared for making the assignments. Details of the chemical and isotopic manipulations will be described elsewhere.

The hf coupling constants obtained by isotopic substitution are listed in Table 1. These constants successfully reproduce the esr spectrum. All but one of the assignments agree satisfactorily with, and in fact are in part based on, theoretical predictions of the spin density distribution in chlorophyll cation radicals (25). The Chl a^+ and Bchl$^+$ hf coupling constants must be interpreted in terms of the following three groups of protons: (*1*) the peaks with the smallest coupling constants (near the center of the spectrum) (Fig. 1) must arise mainly from the methine protons and/or the proton at position 10; (*2*) the four intense peaks originate from methyl group interactions, the largest hyperfine splittings occurring at the 5a-methyl group; (*3*) the less intense outer wings of the spectrum with the largest hyperfine splittings are associated with protons on reduced pyrrole rings (protons 7 and 8 in Chl *a;* protons 3, 4, 7, and 8 in Bchl).

In Vivo Endor. It is apparent that a comparison between *in vitro* and *in vivo* would be much more cogent if the *in vivo* hyperfine splittings could be assigned independently. Remarkably enough, this can be readily accomplished. *Rhodospirillum rubrum* grown in ^2H_2O on succinic acid-$^1H_4$ as a carbon source produces Bchl containing ^2H at all of the methine positions and at positions 3, 4, 7, and 8. Conversely, *R. rubrum*



		R_1	R_2	R_3	R_4
I	Chlorophyll *a*	$H_2C{=}CH-$	H	CH_3O_2C-	phytyl-
II	Bacteriochlorophyll *a**	CH_3COCH_2-	H	CH_3O_2C-	geranylgeranyl-
III	Methylpyrochlorophyllide *a*	$H_2C{=}CH-$	H	H	methyl

* Contains additional 2 H at positions 3 and 4.

cultured in H_2O on succinic acid-$^2H_4$ biosynthesizes Bchl containing ^1H at positions 3, 4, 7, and 8 and the methine protons (26, 27). In both cases the CH_3 groups contain both ^1H and ^2H. Thus, these experiments directly assign the hyperfine coupling constants of the 1a- and 5a-CH_3 groups of *R. rubrum*. Although it has not yet been possible to produce similar isotope hybrids in green plants, it appears likely that heterotrophic green algae, or even *Chlorella vulgaris* cultured heterotrophically will make it possible ultimately to assign the *in vivo* endor spectra of green plants with the same unambiguities as now are obtained in photosynthetic bacteria.

Comparison of In Vivo and In Vitro Endor. The first comparisons were based only on the hf splittings of corresponding peaks (28, 29). Fig. 2 compares *in vitro* and *in vivo* endor signals from monomer Chl a^+ and Bchl$^+$ with endor signals from the blue-green alga *Synechococcus lividus* and the photosynthetic bacterium *R. rubrum*. It can be seen at once that the differences between *in vivo* and *in vitro* spectra are much greater than any changes produced by chemical alteration of the chlorophylls, and can only be explained by the special pair model. The coupling constant data of Table 1 strongly support the special pair model in the photosynthetic bacteria, as the average "aggregation number" deduced for the photo-reaction center chlorophyll *in vivo* is close to the factor of two required for spin sharing by two Bchl molecules.

For green plants interpretation is rather more complicated, but essentially the same conclusions are arrived at from the available data as for photosynthetic bacteria. These considerably more complex spectra can best be interpreted on the basis that the special pair Chls have side chain methyl groups that do not experience free rotation. The esr linewidth of *C. vulgaris* is broadened by 0.5 ± 0.2 G on cooling from room temperature to 80°K, a result which is consistent with immobilization of the methyl groups. Esr data in *C. vulgaris* highly enriched in ^{13}C shows the chlorophyll macrocycle to be rigid on the esr time scale even at room temperature (6). Immobilization of the rotating methyl groups is consistent with an esr Gaussian line shape, and the relative intensities of the endor lines receive a reasonable interpretation in the the same terms. All of these observations support the view

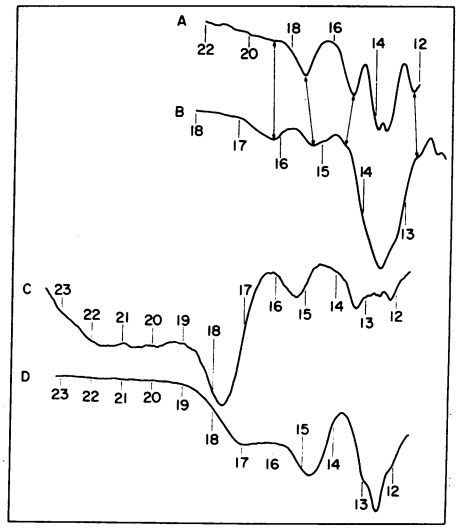


FIG. 2. Comparison of *in vitro* and *in vivo* endor. (A) ^1H Chl *a* in $C^2H_2Cl_2$–$C^2H_3O^2H$ (4:1) oxidized by I_2. Frequency scale (MHz) compressed by a factor of two (T = 108°C); (B) *S. lividus* oxidized by $K_3Fe(CN)_6$. Coupling constants are compared at points connected by lines (T = 108°C); (C) Bchl in $C^2H_2Cl_2$–$C^2H_3O^2H$ (4:1) (T = 15°K); (D) *R. rubrum* oxidized by $K_3Fe(CN)_6$ (T = 15°K).

that green plant endor spectra are best interpreted in terms of special pair chlorophylls in which side chain methyl groups are partially or completely immobilized.

To provide a more quantitative comparison of *in vivo* and *in vitro* endor spectra for Chl *a*-containing organisms, we have compared coupling constants *in vitro* and *in vivo* in Table 1 as indicated in Figs. 2A and B. The comparison between the Chl a^+ *in vitro* hf coupling constants and the *in vivo* data again shows a decrease in the *in vivo* coupling constants by a factor close to 2. The general features of the *in vivo* green plant endor spectra appear to be interpretable only in terms of chlorophyll molecules brought into close proximity, i.e., special pairs.

DISCUSSION

We conclude that in the organisms we have studied here the doublet free radical produced in the primary act of photosynthesis arises from a special pair of chlorophyll molecules. This pair of chlorophyll molecules is special in the following ways: (*1*) the members of the pair share approximately equally the unpaired electron as observed by esr or endor; (*2*) a special geometry exists which allows this spin sharing and which results in an optical red shift; (*3*) the geometry in the special pair is *not* the same as that of chlorophyll dimer, Chl_2, a species produced by keto C=O···Mg interactions and characteristically present in dry benzene or carbon tetrachloride solution (30, 31). Based on the known photo-activity of the chlorophyll–water adduct absorbing at 740 nm already referred to, a water molecule could provide the required orientation for the two chlorophyll molecules in the special pair. However, bifunctional ligands (18) other than water may be involved, or even a protein structural matrix conceivably could be implicated as the required orienting mechanism. The endor data in this communication are entirely compatible with our earlier proposal (8) that the geometry of the repeating unit in the chlorophyll–water adduct, i.e.,

the Chl·H_2O·Chl unit, has the correct properties required for the *in vivo* special pair. Fong (32) has suggested a variant of the Chl·H_2O structure for the special pair *in vivo* in which two chlorophyll molecules and two water molecules are arranged in a highly symmetrical fashion. Both the endor data and preliminary data we have obtained on *in vivo* and *in vitro* bacterial triplet states suggest significant deviations from axial symmetry the triplet data appear to be compatible with the Chl·H_2O·Chl model. It is clear that in the special pair formulation (8, 20, 21) one chlorophyll molecule may act as acceptor for the other as electron donor, possibly by way of an initial triplet state, making a Chl *a* or Bchl molecule the primary acceptor in the light conversion step (21). The special pair model for photo-reactive chlorophyll in combination with studies on the nature of antenna chlorophyll (33) now makes possible a comprehensive model for plant and bacterial photosynthesis (34, 35).

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