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ELECTRON SPIN RESONANCE IN ^{13}C-LABELED CHLOROPHYLL AND ^{13}C-LABELED ALGAE*

JAMES R. NORRIS, ROBERT A. UPHAUS AND JOSEPH J. KATZ
Chemistry Division, Argonne National Laboratory, Argonne, Ill., 60439 (U.S.A.)
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SUMMARY

ESR signals have been recorded from algae and chlorophyll *a* highly enriched in ^{13}C. A theoretical treatment of the effects on line shape to be expected from ^{13}C substitution has been developed. The ^{13}C isotope effect in the line shape of ESR photosignal I in ^{13}C-labeled algae is shown to be consistent with delocalization of the spin over a special pair of chlorophyll molecules. The anisotropy of ^{13}C ESR signals allows the conclusion to be drawn that reaction center chlorophyll in photosynthesizing algae is held in a rigid matrix.

INTRODUCTION

There is at present general agreement that the photo-induced electron spin resonance (ESR) signal associated with the primary light conversion act in photosynthesis arises from the photo-oxidation of certain special chlorophyll molecules in the photosynthetic reaction centers[1]. These special reaction center chlorophylls, designated P700 (in green plants) and P870 (in purple photosynthetic bacteria) because of their anomalous redshifted optical absorption maxima, are presumed to give rise to the free radical species, Chl^+ and $BChl^+$, during transient oxidation of the pigments. Concomittant with the appearance of the free radical signal is a (reversible) photobleaching at 700 nm. There are, however, serious difficulties in identifying P700 as a monomeric chlorophyll species. Thus, the line width of the free radical signal from Chl^+ or $BChl^+$ produced in the laboratory and known with certainty to be monomeric chlorophyll species[2,3] are much broader than the reversible photo-ESR signals observed in the plant. Monomeric chlorophyll species in the laboratory, moreover, absorb light as wavelengths considerably shorter than 700 nm. The anomalous features of the ESR and optical properties of P700 reaction center chlorophyll in the plant have been attributed to chlorophyll "aggregation" of an unspecified nature[4], to chlorophyll–lipid or chlorophyll–protein interactions[5], or to perturbations in the chlorophyll π systems resulting from unspecified changes in the environment[2], but these explanations are not very specific.

Recent investigations on chlorophyll in the laboratory make it possible to characterize a number of distinct chlorophyll species, and a consideration of these

Abbreviations: BChl, bacteriochlorophyll; Chl, chlorophyll.

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puts the dilemma in sharper focus. Spectroscopic investigations, including absorption spectroscopy in the visible* and infrared[6-9], proton and ^{13}C magnetic resonance[8,10-12] and molecular weight determinations by vapor phase osmometry[13] and ultracentrifugation**, all support the view that the chlorophyll molecule has an unusual combination of electron donor and acceptor properties, and that these factors are decisive in determining the state of chlorophyll in solution[14]. The keto C=O function of Ring V of chlorophyll can serve as an electron donor to the Mg of another chlorophyll molecule when other electron donors are absent to generate chlorophyll dimers and higher oligomers[13,15]. Monofunctional ligands such as pyridine, tetrahydrofuran, methanol, acetone, and the like form monomeric chlorophyll species, $Chl \cdot L_1$ and $Chl \cdot L_2$, that absorb light at short wavelengths and yield broad ESR signals. Bifunctional ligands such as dioxane[16], pyrazine, 1,4-diazabicyclo(2.2.2)octane*, and particularly water[17,18] form species of the general type Chl Chl–L–Chl Chl–L–Chl Chl– or –Chl–L–Chl–L–Chl–L– that are markedly red-shifted and which possess either broad or very narrow ESR signals on oxidation. Chlorophyll dimer, Chl_2, or oligomer, $-Chl \cdot Chl \cdot Chl \cdot Chl-$, are modestly red-shifted and have broad ESR signals.

The reversible photo-ESR signal associated with the light conversion act (Signal I of Commoner *et al.*[19]) appears to be generated from a chlorophyll species absorbing near 700 nm and has the free electron g value 2.0025, a peak-to-peak line width (ΔH) of about 7.5 g, and a Gaussian line shape with no hyperfine structure. The various chlorophyll species have a broad range of absorption maxima that extends from 662 to 740 nm, and ESR line width ranging from less than 1 gauss to 10 gauss. None of the chlorophyll species so far characterized have exactly the properties of P700 reaction chlorophyll.

A red-shift in the electronic transition spectra occurs only in chlorophyll species containing more than one chlorophyll molecule. Detailed examination of visible absorption spectra by computer deconvolution methods provides good reason to suppose that the bulk of the chlorophyll in the plant, the so-called antenna or light-harvesting chlorophyll, has a structure very similar to, if not identical with, the chlorophyll oligomer, $(Chl_2)_n$ (ref. 6). The chlorophyll–water adduct, $(Chl \cdot H_2O)_n$, when photooxidized to the free radical, $(Chl \cdot H_2O)_n^+$, has a very narrow ESR signal[20], and thus the structural arrangement characteristic of the $(Chl \cdot H_2O)_n$ species[18] can be used to account for the narrowing in the plant ESR Signal I. The extreme narrowness of the ESR signal in $(Chl \cdot H_2O)_n$ has been explained by spin delocalization over the entire micelle[20-22]. It is important to note that spin delocalization does not appear to occur in any chlorophyll–bifunctional ligand adduct other than water. In chlorophyll dimer, oligomer, or micellar species with other bifunctional ligands the ESR signal is broad, with a value close to that of the monomeric chlorophyll free radical species. This implies that spin exchange is slow or does not occur in these species. Based on these considerations, we advanced a model for P700 in which the free electron is delocalized over a special pair of chlorophyll molecules, $(Chl\ H_2O\ Chl)^+$, with the two chlorophyll molecules oriented as in the $(Chl \cdot H_2O)_n$ adduct[22,23]. Calculation shows that delocalization of the spin over just two chlorophyll molecules positioned as in the $(Chl \cdot H_2O)_n$ adduct provides just the right amount of ESR line width narrowing to account for the *in vivo* signal and for a red-shift in the visible absorption spectrum.

* T. M. Cotton, K. Ballschmiter and J. J. Katz, unpublished work.
** T. M. Cotton, W. A. Svec and J. J. Katz, unpublished work.

Water can provide the required structural orientation, but any bifunctional ligand or even a structural matrix that assures the proper orientation and overlap of the two chlorophyll molecules can similarly account for the narrowing of the ESR line width and for an optical red-shift[22].

The ability to grow living organisms with unusual isotopic composition makes it possible to employ the isotope effect on the ESR line shape in both *in vivo* and *in vitro* situations[24] to aid in the interpretation of the ESR spectra. In previous studies on chlorophylls, algae, and bacteria, we found that the relationship

$$\Delta H_{in\ vivo} \cong \Delta H_{in\ vitro}/\sqrt{2} \qquad (1$$

accounts satisfactorily for the *in vivo* ESR line width[22]. Here $\Delta H_{in\ vivo}$ is the peak-to-peak ESR line width of an *in vivo* system and $\Delta H_{in\ vitro}$ is the line width of the corresponding chemically oxidized *in vitro* monomeric chlorophyll. The relationship described in Eqn 1 is accurately followed by a considerable variety of photosynthetic organisms, including those in which ^1H had been completely replaced by ^2H. The relationship of Eqn 1 implies that the unpaired electron in the photosynthetic reaction center is shared by two molecules of chlorophyll, *i.e.* $(Chl\ H_2O\ Chl)^+$.

In the present work we extend our ESR studies to chlorophyll and living algae highly enriched in ^{13}C (ref. 25). Isotope substitution of ^{12}C by ^{13}C provides a new system for an ESR study of P700 and a test of our model of P700. Additionally, ^{13}C provides a molecular probe of P700 and chlorophyll quite different from the hydrogen probe. ^{13}C systems will have larger ESR line widths than the corresponding natural abundance ^{12}C systems. We found previously that the ESR signal of ^{12}C-labeled chlorophyll is essentially isotropic[25], as little difference existed between the line shape in liquid or solid media, an observation which, incidentally, confirms the small anisotropic contribution of hydrogen to the ESR signal. Nevertheless, we know from studies of fully deuterated systems that ^1H accounts for no less than approx. 85 % of the ESR line width in organisms or chlorophyll of normal isotopic composition; nitrogen, therefore, accounts for only a very small fraction of the ESR line width. In ^1H–^{12}C and ^2H–^{12}C systems, the line shapes are Gaussian, which may be taken as an indication that the line width arises from interactions of the unpaired electron with many hydrogen atoms as opposed to only a few hydrogen atoms. In any event, the ESR signal of chlorophyll that contains ^{13}C at natural abundance (1.1 %) or ^2H is not sensitive to the motional properties of the macrocycle. In contrast, ^{13}C coupling constants usually exhibit hyperfine anisotropy, and accordingly, highly enriched ^{13}C-labeled chlorophyll might be expected to show increased hyperfine anisotropy. This anisotropy provides another parameter for distinguishing between ESR signals. It also allows investigation of rotational freedom of the chlorophyll macrocycle in various environments, as well as providing a new system for investigating the ESR spectra of chlorophyll *a in vitro* and in P700. Our expectations are seen below to be amply fulfilled.

EXPERIMENTAL

The unicellular blue-green alga *Synechococcus lividus* was used in our studies of the *in vivo* photo-induced ESR signals. It was grown autotrophically on 96 % $^{13}CO_2$ as previously described[25]. Pure ^{13}C-labeled chlorophyll *a* was prepared[26] from

cultures of the blue-green alga, *Phormidium luridum*, likewise enriched in ^{13}C. In no case was the initial inoculum more than 2 % of the final cell mass. This is a very important precaution, because an isotopically mixed population gives ESR signals much more difficult to interpret quantitatively. Preliminary studies indicated that the actual content of incorporated ^{13}C was verified by combustion of tissue and mass spectrometric determination of isotopic ratios. All ESR techniques were as described previously[25]. It was found essential in recording the net *in vivo* Photosignal I to remove the contribution of spurious photosignals and the dark signal background. This was achieved by repetitive light–dark addition–subtraction cycles, using a Varian C-1024 computer of average transients. The *in vitro* signal was digitized to facilitate line shape analysis, and all calculations were carried out on a Sigma 5 computer.

TABLE I

ESR LINE WIDTHS OF CHLOROPHYLL *a* SPECIES OF VARIOUS ISOTOPIC COMPOSITIONS

System *	*Solvent system*	λ_{max} (*nm*)	*Oxidant*	ΔH ** (*gauss*)
^{13}C-Chl·L				15.2 ± 0.5
^{1}H-Chl·L	***	663	I_2	9.3 ± 0.3
^{2}H-Chl·L				3.8 ± 0.2
^{13}C-(Chl_2)				13.5 ± 0.5
^{1}H-(Chl_2)	Carbon tetrachloride	665, 678	I_2	9.0 ± 0.5§
^{2}H-(Chl_2)				4.5 ± 0.5
^{13}C-$(Chl_2)_n$				15.3 ± 0.5
^{1}H-$(Chl_2)_n$	Film	665, 678	I_2	10.0 ± 0.5
^{2}H-$(Chl_2)_n$				5.0 ± 0.5
^{13}C-$(Chl_2)_n$				15.4 ± 0.5
^{1}H-$(Chl_2)_n$	Hexadecane	665, 678	I_2	9.0 ± 0.5
^{2}H-$(Chl_2)_n$				4.8 ± 0.3
^{13}C-$(Chl\cdot H_2O)_n$				3.0§§
^{1}H-$(Chl\cdot H_2O)_n$	Film	743	I_2	1.5 ± 0.5
^{2}H-$(Chl\cdot H_2O)_n$				0.8 ± 0.3
^{13}C-$(Chl\cdot H_2O)_n$				2.5§§
^{1}H-$(Chl\cdot H_2O)_n$	Film	743	Light	1.8 ± 0.4
^{2}H-$(Chl\cdot H_2O)_n$				0.8 ± 0.2
^{13}C-$(Chl\cdot H_2O)_n$				2.0§§
^{1}H-$(Chl\cdot H_2O)_n$	Hexadecane	743	I_2	1.0 ± 0.3
^{2}H-$(Chl\cdot H_2O)_n$				0.5 ± 0.2
^{13}C-$(Chl\cdot H_2O)_n$				1.0 ± 0.2
^{1}H-$(Chl\cdot H_2O)_n$	Hexadecane	743	Light	0.7 ± 0.2
^{2}H-$(Chl\cdot H_2O)_n$				0.5 ± 0.1

* The chlorophyll system of usual isotopic composition is listed in the middle. The prefix indicates which isotope has been substituted.

** All values are recorded at room temperature except that of Chl·L which is given at −170 °C

*** Methylene chloride–methanol at −170 °C Liquid solution value for [^{13}C]Chl·L is 13.2 ± 0.3

§ Relatively small signal sizes are observed.

§§ Values up to 5 gauss have been observed. We believe the ESR line width of [^{13}C]$(Chl\cdot H_2O)_n$ systems are highly sensitive to the size of the aggregate. The larger line width reflects small aggregates.

TABLE II

OBSERVED AND CALCULATED ESR LINE WIDTHS (ΔH) OF Chl·L^+ AND SIGNAL I FOR NATURAL ABUNDANCE ^{13}C AND HIGHLY ENRICHED ^{13}C SYSTEMS

System	*Line shape*	ΔH *(gauss)*	
		Observed *	*Calculated*
^{13}C, ^{1}H-Chl *a*·L	Non-Gaussian	15.2 ± 0.5	—
^{12}C, ^{1}H-Chl *a*·L	Gaussian	9.3 ± 0.3 **	—
^{13}C, ^{1}H-*S. lividus*	Non-Gaussian	13.0 ± 0.5	12.2 ± 0.5 ***
^{12}C, ^{1}H-*S. lividus*	Gaussian	7.1 ± 0.7 **	6.6 ± 0.3 §

* ESR of algae recorded at 23 °C; *in vitro* chlorophyll *a* systems at −160 °C in methylene chloride–chloroform.

** Values obtained from ref. 25.

*** Calculated by Eqn 2.

§ Calculated by Eqn 1

RESULTS AND DISCUSSION

^{13}C-labeled chlorophyll ESR

The results of an ESR study of chlorophyll *a* systems of various isotopic composition are summarized in Table I. In all cases, the ^{12}C–^{2}H system has the narrowest line width, and ^{13}C–^{1}H the largest. The ESR line width, as seen in Table II, is significantly increased by ^{13}C incorporation. Figs 1 and 2 clearly illustrate this pronounced

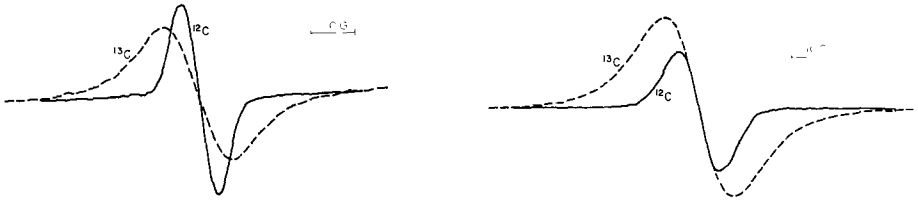

Fig. 1. Comparison of the ESR Photosignal I in living *Synechococcus lividus* at 23 °C. ———, natural abundance ^{13}C; — — —, 96% enriched ^{13}C.

Fig. 2. Comparison of ESR produced by I_2 oxidation of chlorophyll *a* at −150 °C in methanol–glycerol (50·50, v/v). ———, natural abundance ^{13}C; — — —, 96% enriched ^{13}C.

effect for both *in vivo* and *in vitro* chlorophyll ESR signals. The line widths can be divided into lines that are either broader or narrower than the *in vivo* signal. The narrow lines occur only for highly structured and highly red-shifted chlorophyll species. The *in vivo* signal falls in between the two extremes in line widths. Experimentally, this suggests that P700 represents a state of organization intermediate between chlorophyll dimer and the $(Chl \cdot H_2O)_n$ adduct.

In vitro experiments with ^{13}C-labeled chlorophyll show that solid, rigid systems yield line shapes different from those observed in liquid, mobile systems (Fig. 3). The line shape of a rigid *in vitro* system is observed to be very close to that seen *in vivo*, which we take to indicate that reaction center chlorophyll in the plant must be constrained in a fixed matrix.

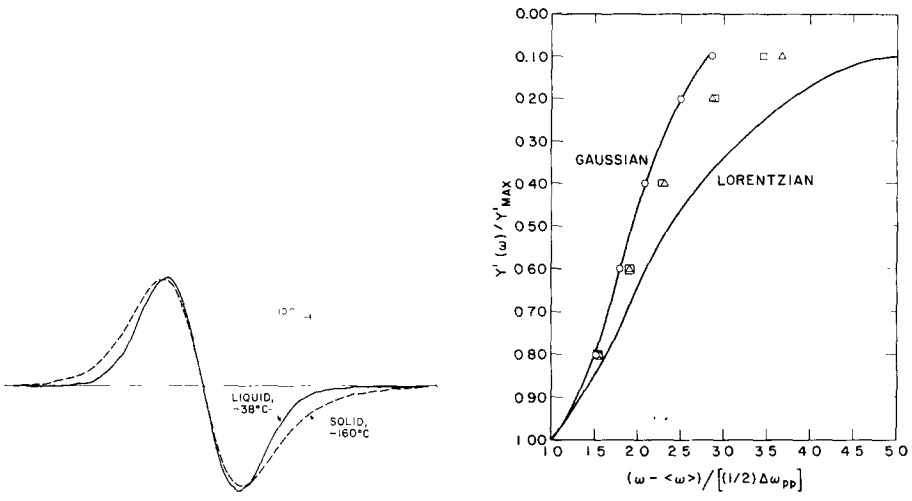

Fig. 3. Comparison of ESR seen in a 96% ^{13}C-labeled chlorophyll liquid solution and as the solid frozen system. ESR signals produced by I_2 oxidation. Solvent system was methylene chloride–methanol (1 : 1, v/v).

Fig. 4 Line shape comparisons for ESR first derivative line shapes. The abscissa is proportional to units of normalized line widths; the ordinate is proportional to units of normalized ESR intensity. Solid lines, theoretical curves: specific points from experimental data, as follows: ○, *in vitro*, liquid solution; □, *in vitro*, frozen solution, △, *in vivo* cells

^{13}C-labeled algae ESR

The line shape of *in vivo* signals, or those seen in ^{13}C-labeled chlorophyll *a* rigid systems, are not of pure Gaussian shape, in contrast to the Gaussian curves found for chlorophyll *a* in freely tumbling solutions or in chlorophyll composed largely of ^{12}C. Deviation from the Gaussian line shape form is observed only in systems highly enriched in ^{13}C. Fig. 4 indicates the nature and extent of the deviation of experimental line shapes from the theoretical curves for pure Gaussian or pure Lorentzian functions.

Deviation from an ideal Gaussian line shape is most probably intrinsic to ^{13}C-containing systems because of large anisotropic ^{13}C hyperfine interactions. This conclusion is supported by a comparison of *in vitro* solid (rigid) and solution (mobile) ESR signals. The existence of anisotropy implies that the photoactive chlorophyll system in the living organism is rigid on the ESR time scale. Thus, we estimate that P700 in the plant must have a rotational correlation time greater than (approximately) 10^{-7} s.

In vivo and in vitro line widths

The above discussion deals with general changes of line shape and line width upon ^{13}C substitution. We now compare the *in vivo* line width with the *in vitro* line width. Previously[22], we calculated the change in line width when an unpaired electron is delocalized over a pair of molecules, *i.e.* when the electron travels rapidly back and forth between the members of the special chlorophyll pair. The calculations for the ^{1}H and ^{2}H cases were very simple because only Gaussian line shapes were considered. This treatment was adequate because all pertinent ESR signals originating from

^{12}C-labeled chlorophyll systems were, in fact, Gaussian. This simple and satisfactory model predicted that for Gaussian line shapes the ESR signal of the pair of chlorophyll molecules is $1/\sqrt{2}$ times smaller than the line width of monomeric chlorophyll species. The *in vivo* Photosignal I in a wide variety of photosynthetic organisms is, in fact, very nearly $1/\sqrt{2}$ times smaller than the corresponding *in vitro* ESR signal obtained from monomeric Chl·L^+ systems.

In contrast, the present study with ^{13}C indicates that chlorophyll systems highly enriched in ^{13}C (approx. $> 90\%$) no longer possess the simple Gaussian line shape, and the analysis given below shows that the $\sqrt{2}$ change in line width is no longer applicable, even if delocalization over a chlorophyll pair does occur. In order to explore the validity of the chlorophyll-pair active center model for ^{13}C-containing systems, we must now calculate the change in line width to be expected upon sharing the unpaired electron between pairs of molecules that give non-Gaussian ESR signals for monomeric Chl·L^+.

In order to treat a general system we consider any monomeric absorption line shape function, $g_m(\boldsymbol{H})$, where $\boldsymbol{H}$ is the magnetic field. Calculation of the change in line width when the unpaired electron spin is delocalized over a pair of monomers is complicated except in the case of Gaussian line shapes. We therefore make the basic assumption that resonance fields in the special pair of monomers occur at positions determined by averaging all possible pairs of monomer resonance fields, an assumption similar to the one made previously[22]. Under these conditions

$$g_P(\boldsymbol{H}) \alpha \int_0^\infty g_m(\boldsymbol{H} - X) g_m(\boldsymbol{H} + X) \mathrm{d}X \tag{2}$$

where $\mathrm{d}g_P(H)/\mathrm{d}H$ is the first derivative absorption line shape for the special pair. If one member of the pair is resonant at field $\boldsymbol{H} - X$ and the other is resonant at field $\boldsymbol{H} + X$ then the special pair is resonant at field $\boldsymbol{H}$. The product of line shape functions in the integral gives the relative number of pairs of molecules resonant at field $\boldsymbol{H}$. The integral sums the number of pairs that are in resonance at field $\boldsymbol{H}$ for all possible values of X. Eqn 2 gives the above mentioned $1/\sqrt{2}$ narrowing effect for the case that $g_m(\boldsymbol{H})$ is a Gaussian, but it also yields the more surprising result that the line width changes not at all when $g_m(\boldsymbol{H})$ is Lorentzian.

The treatment given here shows that for Lorentzian line shapes the line width remains constant upon delocalization. The treatment of the Gaussian line shape predicts the signal from the chlorophyll pair to be narrowed by a factor of $\sqrt{2}$ as compared to the signal from the monomer. In the experimentally observed line shape for the ^{13}C-labeled chlorophyll systems of interest, a line shape of intermediate character is found. The ESR signal is characterized by highly extended intensity in the wings of the spectrum. A computer calculation using Eqn 2 on the experimentally determined line shape for our *in vitro* ^{13}C-labeled Chl·L system predicts that the special pair will have a signal approx. 1.26 times narrower in line width than the value for the monomer. Thus, for the line shape typical of ^{13}C-labeled chlorophyll, we expect that the line width will decrease by a factor of approx. 1.26 in going from monomeric chlorophyll, Chl·L^+, to a pair of chlorophylls, (Chl H_2O Chl)$^+$. Thus, we expect for the ^{13}C case

$$\Delta \boldsymbol{H}_{in\ vivo} \cong \Delta \boldsymbol{H}_{monomer}/1.26 \tag{3}$$

The results given in Table I are in good agreement with this relationship. It is

evident, also, that a parallel exists with the values given in $^{1}H + {}^{2}H$–^{12}C systems, in that the predicted values for signals from a pair of chlorophyll molecules are always somewhat less than those observed in *in vivo* systems.

Our proposed model for photosynthetic chlorophyll[25] is thus seen to be supported by the present work. First, the line shape for *in vivo* Signal I and rigid *in vitro* $Chl \cdot L^{+}$ are changed from the Gaussian line shape in a very similar way by ^{13}C substitution. This result in addition indicates that P700 is rigid on an ESR time scale, an observation that is certainly consistent with the hypothesis that chlorophyll is, in fact, the origin of the *in vivo* signal. Second, the measured *in vivo* line width value agrees with the value predicted for an ESR line with a line shape intermediate between Gaussian and Lorentzian produced by an electron delocalized over two special chlorophyll molecules in the photosynthetic reaction center.

The experiments reported here emphasize the value of ^{13}C substitution in biological ESR studies, especially for systems that give ESR signals without hyperfine structure. In earlier work with chlorophyll *a* the *g*-value, the line width, and the line shape were the only parameters that could be measured. Because all line shapes were Gaussian, little additional information could be derived. In the case of ^{13}C-labeled chlorophyll systems, however, the additional anisotropy associated with the line shape becomes significant, and additional information can be deduced. We have learned with interest that the recent ESR results of McElroy *et al.*[26] in photosynthetic bacteria appear to be accommodated satisfactorily by the model described here.

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