The Cation Radicals of Free Base and Zinc Bacteriochlorin, Bacteriochlorophyll, and Bacteriopheophytin

(electron spin resonance/optical spectra/oxidized pheophytins/free radicals/photosynthesis)

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ABSTRACT   One-electron oxidation of zinc tetraphenylbacteriochlorin and its metal-free base yielded stable cation radicals. Electron spin resonance hyperfine splittings were assigned by selective deuterations. These results indicate that the protons of the saturated rings of the bacteriochlorins carry large spin densities, in accord with molecular orbital calculations. Comparison *in vitro* of the optical spectra of bacteriochlorins and their cation radicals with those of bacteriochlorophyll show close correspondence and suggest that the electron spin resonance data from the former may also prove a guide to the biological molecule. The surprising similarity in properties between the radicals of free base and zinc bacteriochlorins is maintained in the chlorophylls: cation radicals of bacteriopheophytin and methyl pheophorbide (the free bases of bacteriochlorophyll and methyl chlorophyllide, respectively) exhibit electron spin resonance properties similar to those of their magnesium-containing derivatives. The possibility that metal-free chlorophylls participate in photosynthesis is discussed.

The primary photochemical step of intact photosynthesizing systems, extracted reaction centers, and chlorophylls involves electron ejection from chlorophyll a (Chl) in green plants and algae and from bacteriochlorophyll (BChl) in purple bacteria (1–9). The resultant oxidized chlorophylls exhibit properties characteristic of π-cation radicals (7, 8), i.e., the unpaired electron is delocalized over the π system of the aromatic molecule. No electron spin resonance (ESR) hyperfine structure has ever been resolved for the chlorophyll radicals (2, 7, 10–13). The singlet ESR signals observed narrow significantly with deuterochlorophyll (2, 7, 12) and broaden upon ^{13}C substitution (11, 12), which, while confirming the π-radical nature of the oxidized molecules, yields little additional information about the spin-density distribution or the reactive sites of the chlorophylls. Electron-nuclear double resonance (14) has been applied to bacteriochlorophylls *in vitro* and *in vivo*. The results (15, 16) support the hypothesis regarding reaction centers (7), that the photoactivated BChl is a bridged dimeric cation, $(BChl)_2^{+\cdot}$, but they do not yield sufficient information to specify the sites of spin delocalization.

In contrast to the ESR singlets from $Chl^{+\cdot}$ and $BChl^{+\cdot}$ (or from $Chl_2^{+\cdot}$ and $BChl_2^{+\cdot}$), oxidized zinc tetraphenyl-

Abbreviations: Chl, chlorophyll; BChl, bacteriochlorophyll; Ph, pheophytin or methyl pheophorbide; BPh, bacteriopheophytin; TPBC, tetraphenylbacteriochlorin; TPP, tetraphenylporphyrin; ESR, electron spin resonance; ΔH, linewidth, peak to peak, of first derivative singlet ESR signal; a_{H_x} or A_{H_x}, proton hyperfine splitting constant at position-X.
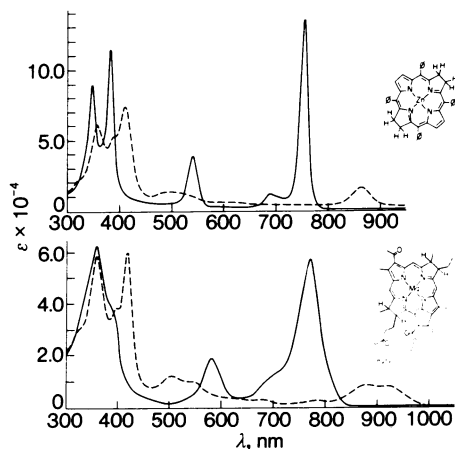
bacteriochlorin ($ZnTPBC^{+\cdot}$) yields hyperfine structure that can be readily assigned, by selective deuterations, to specific positions in the radical. BChl is a modified bacteriochlorin (tetrahydroporphyrin); comparison of its electronic spectra, both as BChl and $BChl^{+\cdot}$, with those of ZnTPBC and ZnTPBC$^{+\cdot}$ shows sufficient correspondence of spectral features to suggest that an analogy between their electronic properties is valid. Hence, the electron delocalization determined from ESR hyperfine structure of $ZnTPBC^{+\cdot}$ and its deuterated derivatives should also provide a model for that of $BChl^{+\cdot}$.

BChl and bacteriochlorin exhibit an unusual property: unlike the major optical shifts observed between metalloporphyrins and porphyrins, complexation of zinc or magnesium by the free bases of these pigments causes little change in optical spectra. Reaction centers contain significant amounts (17–22) of bacteriopheophytin (BPh), the free base of BChl. What is its role, if any, and has it been overlooked in the optical and ESR studies of the primary photosynthetic step because of the similarity of its properties to those of BChl? We present here optical and ESR data on bacteriochlorin, bacteriopheophytin, and methyl pheophorbide (the free base of methyl chlorophyllide) in an attempt to answer these questions.

EXPERIMENTAL

BChl (from *Chromatium*), Chl, and derivatives were prepared by standard techniques (23, 24). The purified pigments exhibited optical spectra that agreed with published data (25). *meso*-Tetraphenylbacteriochlorins (H₂TPBC) were prepared by the diimide method (26). The appropriately deuterated porphyrins (27) were reduced with toluenesulfonylhydrazine in the dark under argon. The resulting mixture of chlorin and bacteriochlorin was separated by chromatography on silica gel, and the eluent was recrystallized from benzene. ZnTPBCs were prepared by reacting dry zinc acetate with H₂TPBC in dry pyridine in the dark under argon. ZnTPBC was precipitated from hot deoxygenated water and dried under reduced pressure. The deuterium content of $ZnTPBC_{d20}$ and $d28$ was about 97% by mass spectral analyses. All optical and ESR spectra were obtained under vacuum or under argon. The techniques for chemical or electrochemical oxidation have been described (8, 27, 28).

RESULTS

$ZnTPBC^{+\cdot}$. Electrochemical oxidation of ZnTPBC ($E_{1/2}$ = 0.18 V against aqueous saturated calomel electrode) in CH_2Cl_2

FIG. 1. Optical absorption spectra, in CH_2Cl_2, of (*top*) Zn-TPBC (——) and the cation radical ZnTPBC$^+$· (– – –); (*bottom*) BChl (——) and the cation radical BChl$^+$· (– – –). Values of ε have been multiplied by 10^{-4}.

requires one electron and yields the optical spectra shown in Fig. 1 *top*. The reaction is reversible: one-electron reduction regenerates better than 95% of the original bacteriochlorin. For comparison, the spectral changes observed during a similar oxidation of BChl are displayed in Fig. 1 *bottom*.¶ The changes are clearly parallel and indicate that the electronic transitions observed primarily reflect the tetrahydroporphyrin framework common to both molecules.

The ESR spectrum of ZnTPBC$^+$· shows well-resolved hyperfine structure (Fig. 2). Identical spectra are obtained by three different oxidation methods (27, 28): (*a*) *in situ* electrochemical oxidation, (*b*) electron transfer to the oxidized salt of μ-oxo-bis(tetraphenylporphyrin iron(III)): (FeTPP)$_2$O$^+$-ClO$_4^-$ + ZnTPBC → (FeTPP)$_2$O + ZnTPBC$^+$·ClO$_4^-$ (the iron compounds show no ESR spectra at room temperature), and (*c*) electron transfer to the cation radical of zinc tetraphenylporphyrin: ZnTPP$^+$·ClO$_4^-$ + ZnTPBC → ZnTPP + ZnTPBC$^+$·ClO$_4^-$. In the last case, integration of the ESR signals shows that the number of spins is conserved (±10%) and thus further establishes that one-electron transfer has occurred. Splitting constants can be assigned with the aid of selective deuterations: deuteration of the phenyl groups causes only minimal changes of the spectra (Fig. 2) and indicates only small contributions from the phenyl groups (computer simulations of the spectra limit these contributions to less than 0.05 G). Deuteration of all positions except the four protons at 2, 3, 12, and 13 yields the spectrum shown in Fig. 3 and confirms that those protons cause the large splittings observed. These conclusions are reflected in the computer-simulated spectra shown in each figure. The computed splitting constants are a_{H_2} = 7.50 G, a_{H_7} = 1.20 G, and a_N = 1.20 G in CH_2Cl_2. In chloroform, the corresponding numbers are 7.33, 1.22, and 1.22 G. (Simulations that do not include the smaller hydrogen splittings *and* nitrogen splittings yield less satisfactory spectra.) These assignments are supported by SCF molecular orbital calculations (28), which yield the spin densities shown in Table 1 and which predict correctly the observed trends of ESR splittings, although not the absolute values. a_{H_2} Reflects (31) the spin density, ρ_1, on the α carbon

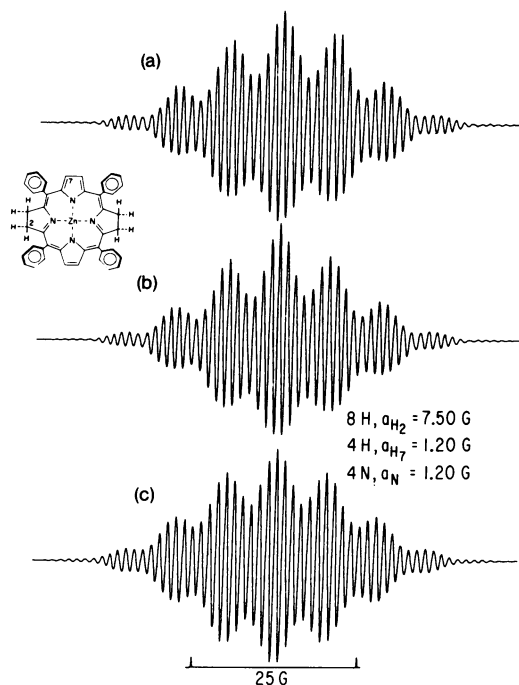¶ The BChl$^+$· spectra agree with those published (29, 30).

FIG. 2. Second derivative ESR spectra (g = 2.0026), in CH_2Cl_2, of (*a*) ZnTPBC$^+$·, (*b*) ZnTPBC$^+$·d_{20}, (deuterated phenyl groups), (*c*) simulation using the splitting constants shown.

8 H, a_{H_2} = 7.50 G
4 H, a_{H_7} = 1.20 G
4 N, a_N = 1.20 G

25 G

(C-1): $a_{H_2} \cong \rho_{C_1}$ (K cos^2 θ) where θ is the dihedral angle between the 2_{p_z} orbital of C-1 and the plane defined by C-1, C-2, and H-2. A more rigorous equation, derived for cation radicals (32), uses a_{H_2} = ρ_{C_1} (−9.2 + 96.7 cos^2 θ). A_{H_7} is governed by the McConnell relationship $a_{H_7} \cong$ 27 ρ_{C_7} and $a_N \cong$ 31 ρ_N − 4 $\rho_{C_1 or 6}$ (27, 28).

H₂TPBC$^+$·. The spectra obtained on electrochemical oxidation ($E_{1/2}$ = 0.40 V) of H₂TPBC in CH_2Cl_2 are shown in Fig. 4. One electron is transferred, as measured by coulometry or exchange with ZnTPP$^+$·ClO$_4^-$. The ESR spectra of selectively deuterated derivatives in CH_2Cl_2 are shown in Figs. 5 and 6. As for ZnTPBC$^+$·, the splitting constants vary with solvent: in CH_2Cl_2, a_{H_2} = 8.11, a_{H_7} = 1.19, and a_N = 1.19 G. In CHCl₃, the corresponding values are 7.87, 1.18, and 1.18 G. The interaction with the inner hydrogens (N–H) appears too
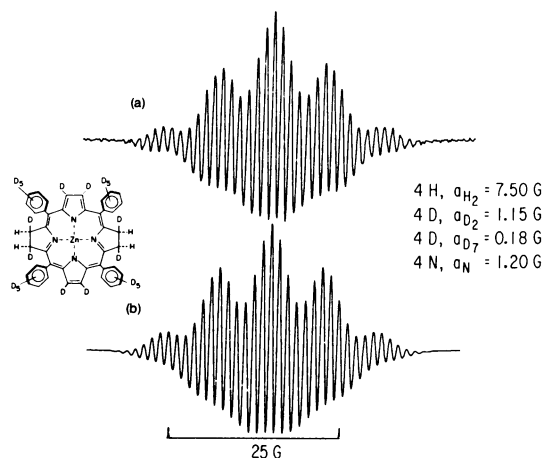

FIG. 3. Second derivative ESR spectra in CH_2Cl_2 of (*a*) ZnTPBC$^+$·d_{28} and (*b*) simulation (a_D/a_H = 0.1535).

4 H, a_{H_2} = 7.50 G
4 D, a_{D_2} = 1.15 G
4 D, a_{D_7} = 0.18 G
4 N, a_N = 1.20 G

25 G
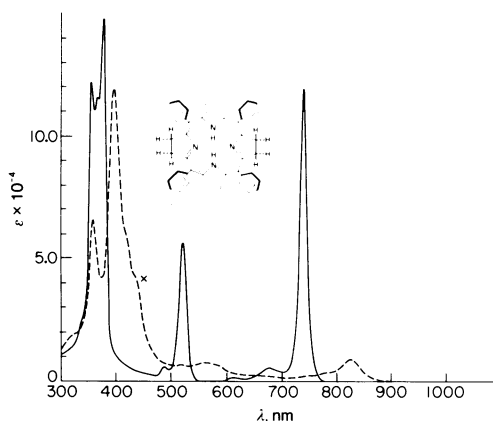
FIG. 4. Optical absorption spectra, in CH_2Cl_2, of H_2TPBC (———) and $H_2TPBC^{+\cdot}$ (— — —). ($\times$ = impurity).

FIG. 5. Second derivative ESR spectra (g = 2.0026), in CH_2Cl_2, of (a) $H_2TPBC^{+\cdot}$, (b) $H_2TPBC^{+\cdot}d_{20}$. (c) Simulation using the splitting constants shown.

small to be detected. In an attempt to exchange these hydrogens (33), $CDCl_3$ was used instead of $CHCl_3$ as solvent: no changes in the ESR spectra of the radical were observed even after several days. The general features of the experimental unpaired spin densities are again in accord with molecular orbital calculations; the calculated spin densities for H_2T-$PBC^{+\cdot}$ are shown in Table 1. The experimental and theoretical results thus establish, as for $ZnTPBC^{+\cdot}$, that the sites of high-spin densities are $C_4 > C_7 > C_5$.

Radicals Derived from Demetallated Chl and BChl. Oxidation of methyl pheophorbide a, the free base parent of methyl chlorophillide a, proceeds (34) at 0.86 V in CH_2Cl_2 to yield the radical shown in Fig. 7. At 25°C the singlet spectrum, g = 2.0026 (±0.0001), is 9 gauss wide and comparable to the signals observed in the same solvent for the cation radicals of Chl and ethyl chlorophyllide (8). The distinguishing features are the higher oxidation potential ($E_{1/2}$ = 0.52 V for Chl), and the lower stability, ($Ph^{+\cdot}$ decays an order of magnitude faster than $Chl^{+\cdot}$ at room temperature in CH_2Cl_2). Further, upon oxidation, the 670-nm absorption band of Ph is partially bleached and a new band appears at 775 nm.[||] A radical of pheophytin has been obtained, at 77°K, by photooxidation in the presence of tetranitromethane (35). It is also characterized by g = 2.0025, ΔH = 9.8 G, partial bleaching of the 670-nm band, and a peak at 775 nm.

Electrochemical oxidation of BPh in CH_2Cl_2 at 0.80 V causes optical changes that parallel those observed for H_2T-PBC (Fig. 8). The reaction is reversible; 90% of the initial BPh is regenerated upon reduction of the radical.

Oxidation of bacteriopheophytin yields the ESR spectrum shown in Fig. 9. The gaussian singlet is 14.4 gauss wide, peak-to-peak, in CH_2Cl_2 and 14.0 G in $CHCl_3$,[**] slightly wider than the values of 13.2 G and 12.8 G obtained for $BChl^{+\cdot}$ in the same solvents.[††] For both compounds g = 2.0025 (±0.0001). For the first time in a cation radical derived from a chloro-

phyll, there is evidence of hyperfine splittings of about 1 G. Again, the pheophytin is harder to oxidize ($E_{1/2}$ = 0.72 compared to 0.40 for BChl in CH_2Cl_2) and an order of magnitude less stable, at room temperature, than the chlorophyll.

CONCLUSIONS

(*1*) Stable cation radicals of ZnTPBC were prepared and their ESR spectra analyzed. The close similarity of the electronic spectra of BChl and ZnTPBC, both derivatives of tetrahydroporphyrins, and of their radicals suggests that the unpaired spin densities of $BChl^{+\cdot}$ should follow the trend observed in $ZnTPBC^{+\cdot}$. (The fifth ring present in $BChl^{+\cdot}$ and the acetyl group on ring I can be expected to alter spin densities somewhat from those of $ZnTPBC^{+\cdot}$, even if the general features of the delocalization are similar.) McElroy *et al.* (2) have deduced nitrogen splittings, a_N = 1.1 G for $BChl^{+\cdot}$, that compare favorably with a_N = 1.2 G, which we find for $ZnTPBC^{+\cdot}$.

Large splitting constants should be observed for the protons on the saturated positions of rings II and IV of $BChl^{+\cdot}$, followed by interactions with the methyl groups of rings I and

[||] Quantitative optical spectra of $Ph^{+\cdot}$ could not be obtained because of some decay during the time required to obtain the spectra.

[**] The line shape of $BPh^{+\cdot}$ in $CHCl_3$ remains gaussian at $-140°$ but the signal saturates easily.

[††] In methanol, we find ΔH = 12.7 G for $BChl^{+\cdot}$ compared with published values of 12.6–12.8 G (29, 30).
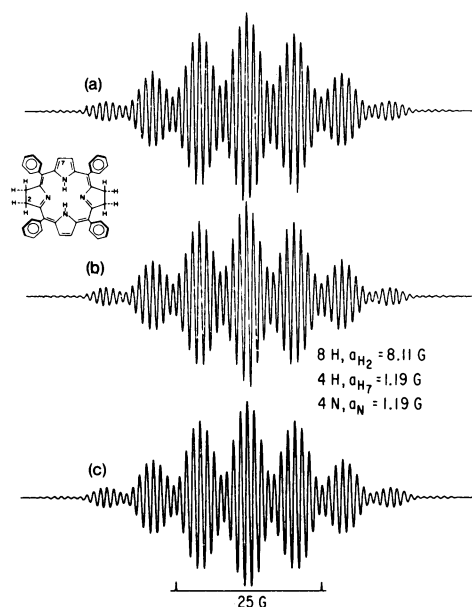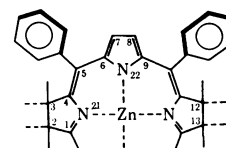
TABLE 1. *Calculated spin densities*

Position	$ZnTPBC^{+\cdot}$ ρ	$H_2TPBC^{+\cdot}$ ρ
21	−0.0071	−0.0170
22	−0.0077	−0.0007 (N–H)
9	0.1157	0.1013
8	0.0292	0.0280
5	−0.0208	−0.0115
4	0.1333	0.1412
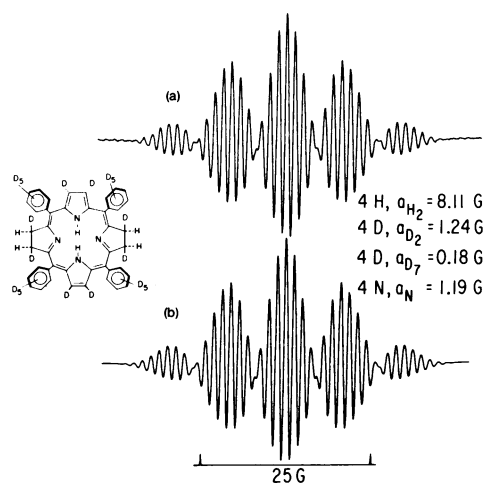
FIG. 6. Second derivative ESR spectra, in CH₂Cl₂, of (a) H₂TPBC⁺·$_{d_{28}}$ and (b) simulation.

III, with the smallest splittings due to the *meso* protons. The large splittings of the β protons thus contribute most to the width, ΔH, of the BChl⁺· ESR signal.‡‡ For ΔH = 13 G, assuming four equivalent protons, a$_H$ for rings II and IV should be about 5 G. (Extension of this argument to Chl⁺·, which has only two, instead of four, β protons, does predict that ΔH for Chl⁺· will be significantly smaller than for BChl⁺·. The experimental ΔH values in CH₂Cl₂ are 13 G for BChl⁺· and 9 G for Chl⁺·.)

(2) Stable radicals of H₂TPBC were also prepared; their existence and the similarity of their spectral properties with those of ZnTPBC⁺· suggested a parallel with demetallated Chl and BChl. We prepared and described such radicals, Ph⁺· and BPh⁺·. The latter exhibits broader ESR linewidths than BChl, in accord with the larger β proton splitting found in H₂TPBC⁺· compared with ZnTPBC⁺·. Optically, the absorption changes observed in the oxidation of BPh reflect those found in H₂TPBC. The bleached red bands are significant features common to all the oxidized tetrahydroporphyrins. Further, all the free bases are harder to oxidize, i.e., the radicals are stronger oxidizing agents than the corresponding magnesium or zinc compounds.§§

The high concentration of BPh (2 BPh: 4 BChl) found in reaction centers (17–22) naturally raises questions about its role in bacterial photosynthesis. Circular dichroism, absorption, fluorescence, and action spectra of reaction centers have led to the conclusion that BPh is strongly coupled to BChl and transfers energy to P870 with high efficiency (22, 37–39). However, on the basis of comparable data, Philipson and Sauer (40) concluded that BPh has *no* function in electron transport while electron-nuclear double resonance results on bacteria, oxidized intact, were inconclusive (16). The data we have presented here lead to the following considerations: If BPh exists as a separate entity in the reaction center and its oxidized form is present in appreciable concentration, then,

‡‡ The other substituents of rings II and IV are two carbon atoms (i.e., γ) removed from the sites of unpaired spin, which will result in splitting constants an order of magnitude smaller than those of the β protons [e.g., a$_{(CH_3)}$ in the cation radicals of zinc tetrapropyl porphyrin (27) and hexaethylbenzene (32)].

§§ This effect of the metal is observed in bacteriochlorins, chlorins, and porphyrins (34, 36).
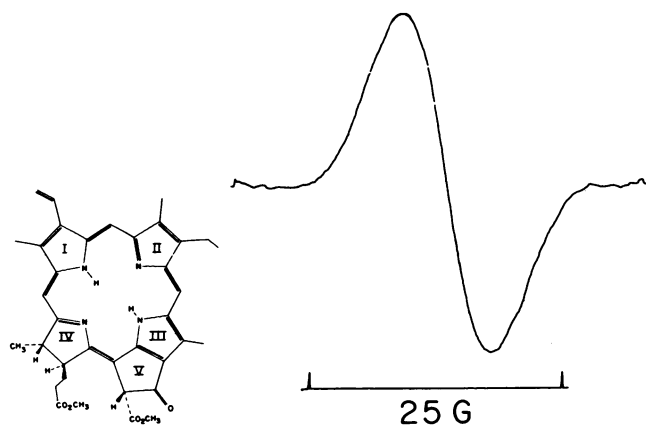

FIG. 7. First derivative ESR spectrum (g = 2.0026), in CH₂Cl₂, of the cation radical of methyl pheophorbide *a*.

based on the solution properties of BPh and BPh⁺·, the center should exhibit a higher oxidation potential than that of BChl, the 750-mm absorption of its BPh component should bleach, and its ESR spectrum should reflect the 14-G width of BPh⁺·. In fact, the ESR signal found upon oxidation *in vivo* has a linewidth of 9.5 G (2, 7). Nor have the other predicted properties apparently been observed, possibly because any BPh⁺· formed would oxidize any accessible BChl due to the
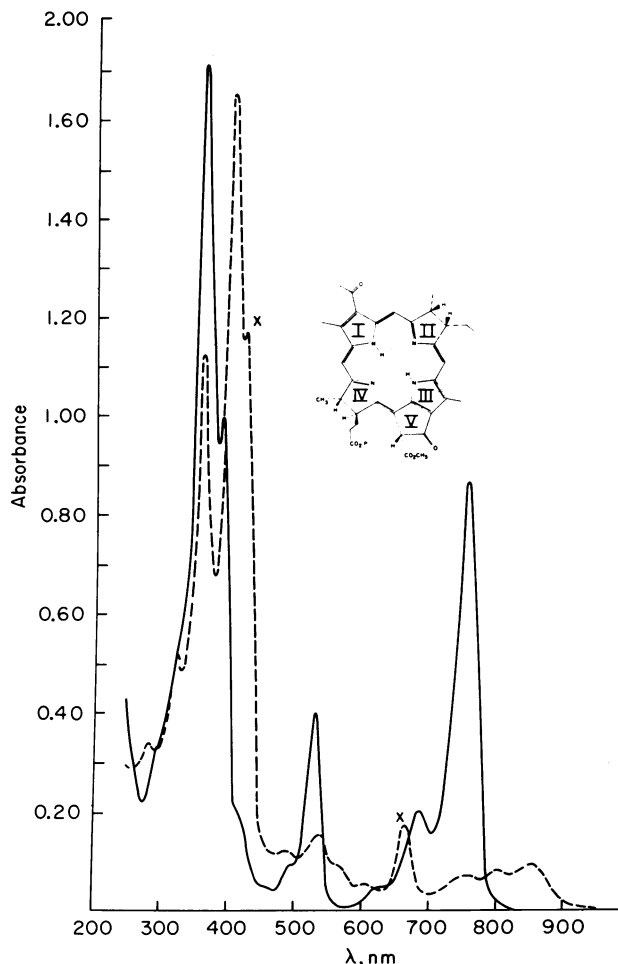

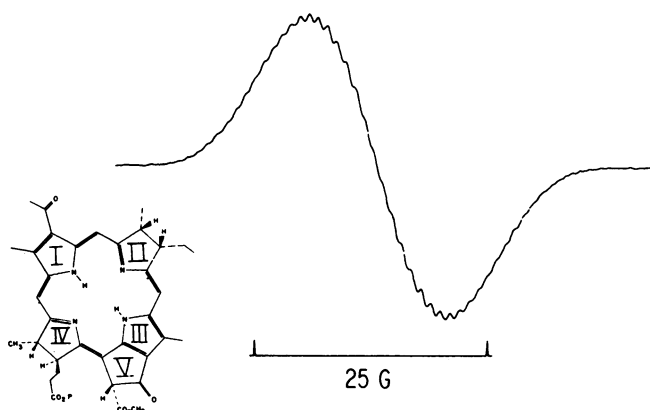FIG. 8. Optical absorption spectra, in CH₂Cl₂, of BPh (———) and BPh⁺· (– – –). (× = impurity).

FIG. 9. First derivative ESR spectrum (g = 2.0025), in CH_2Cl_2, of the cation radical of bacteriopheophytin.

difference in oxidation potentials. On the other hand, if BPh is indeed tightly coupled to BChl or itself (21, 22, 37–39), then the solution properties we have described may be sufficiently altered so as to no longer provide distinguishing characteristics for a BPh radical. In green plants, the presence of pheophytin is less certain, but its function in photosystem II is an intriguing speculation prompted by the higher oxidation potential of Ph compared with Chl and by the similarity between the ESR properties of $Ph^{+\cdot}$ and those of a "chlorophyll" radical ($\Delta H = 8$ G, g = 2.0026) associated (41) with photosystem II. The overlapping absorption bands of Chl and Ph insure exciton transfer from antennae chlorophyll to Ph as well as to Chl.

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