The Biochemical Significance of Porphyrin π Cation Radicals

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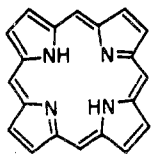
Porphine (1), an aromatic tetrapyrrolic macro-



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cycle,[1] serves as the nucleus for all the naturally occurring porphyrins and related systems. Metalloporphyrins are readily prepared by metal ion displacement of the central nitrogen protons, and all of these systems are characterized by an intense absorption band in the uv (Soret band) and weaker absorptions ($\epsilon \sim 20{,}000$) in the visible and near ir.

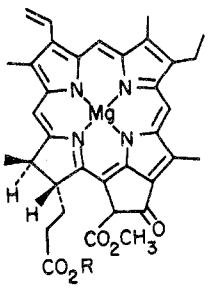
Solid-state structure determinations[2] of porphyrins and metalloporphyrins show that the molecule is not totally rigid and may show ruffling of the skeleton by alternate upward and downward displacement of the pyrrole rings from a planar arrangement. Nonetheless, in an environment free from crystal forces, spectroscopic evidence is consistent with a general planar description of the porphyrin. In metalloporphyrins the metal may be found[2,3] either in or out of this plane, depending upon the metal and its axial ligands.

Interest in porphyrin chemistry is generated by the presence of these species and closely related substances in biological redox processes; of particular importance is their appearance and function in biological oxidations.

The biosphere depends upon photosynthesis to carry out the conversion of photonic into chemical energy as well as to maintain an oxidizing atmosphere for catabolism (conversion of organic compounds to CO_2) which provides the energy necessary to drive endergonic biochemical processes. The classical reaction of photosynthesis requiring chlorophyll a (2) involves CO_2 fixation, *viz.*

$$CO_2 + H_2O \xrightarrow[\text{"chlorophyll"}]{h\nu} [CH_2O] + O_2 \qquad (1)$$

where $[CH_2O]$ is a carbohydrate and water serves as the oxidizable hydrogen donor. A similar reaction occurs in certain photosynthetic bacteria which utilize bacteriochlorophyll (3) and hydrogen donors other than water.



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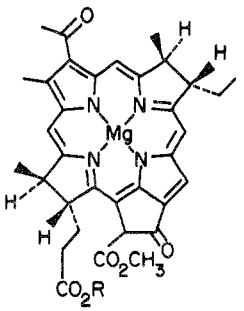

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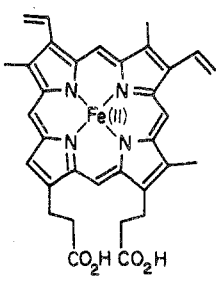
The photosynthetic process divides naturally into light-driven primary reactions[4,5] and the subsequent CO_2 reduction steps studied by Calvin, Bassham, and coworkers.[6] Under the title of primary reactions is included the conversion of light energy into reductants and oxidants and, in the case of green plants and blue-green algae, the chemical and physical states involved in the oxygen-evolving apparatus.

Heme protein participation[7] in oxygen transport, peroxide reduction and disproportionation, the mitochondrial electron transport chain, and drug metabolism (cytochrome P450) stresses the biological importance and diverse roles of the iron porphyrins. Iron protoporphyrin IX (4) (heme) is the prosthetic group of hemoglobin, myoglobin, catalase, peroxi-



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David Dolphin received both his B.Sc. and Ph.D. degrees from the University of Nottingham. In the fall of 1965 he moved to Harvard where he spent a year as a postdoctoral fellow with R. B. Woodward. He then spent 7 years on the faculty at Harvard and in January 1974 joined the faculty at the University of British Columbia. The structure, synthesis, chemistry and biochemistry of porphyrins, vitamin B_{12}, and related macrocycles are the main focus of his research.

Ronald Felton received his B.S. from M.I.T. and Ph.D. in Chemical Physics at Harvard University. He joined the faculty at Georgia Tech in 1968 where he is now an Associate Professor. Professor Felton is interested in the statistical theory of electronic energies as well as the physical properties of porphyrin ions.

(1) J. E. Falk, *Porphyrins Metalloporphyrins*, (1964).
(2) E. B. Fleischer, *Accounts Chem. Res.*, **3**, 105 (1970).
(3) J. L. Hoard, *Science*, **174**, 1295 (1971).
(4) N. I. Bishop, *Annu. Rev. Biochem.*, **40**, 197 (1971).
(5) R. K. Clayton, *Annu. Rev. Biophys. Bioeng.*, **2**, 131 (1973).
(6) M. Calvin and J. A. Bassham, "The Photosynthesis of Carbon Compounds," W. A. Benjamin, New York, N. Y., 1962.
(7) H. R. Mahler and E. H. Cordes, "Biological Chemistry," 2nd ed., Harper and Row, New York, N. Y., 1971, Chapter 15.

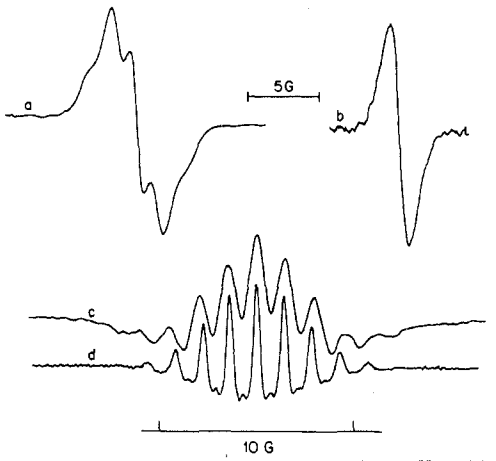


Figure 1. First derivative epr spectra of (a) $[Mg^{II}OEP]\cdot^+ClO_4^-$ and (b) $[Mg^{II}OEP\text{-}meso\text{-}d_4]\cdot^+ClO_4^-$ in methanol at −50°. Second derivative epr spectra of (c) $[Mg^{II}TPP]\cdot^+ClO_4^-$ and $[Mg^{II}TPP\text{-}d_{20}]\cdot^+ClO_4^-$ (perdeuterated phenyl groups) in chloroform.

dase, and many of the cytochromes. The respiratory pigment hemoglobin contains four heme prosthetic groups and is distributed in red blood cells; myoglobin is a monomer found in muscle cells. Both pigments reversibly bind oxygen for use in cellular catabolism. Hydroperoxidases are hemiproteins (iron is present as Fe^{3+} in the resting enzyme) which serve to catalyze the reaction

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (2)$$

in the case of catalase or a peroxidative reaction

$$ROOH + H_2A \xrightarrow{\text{peroxidase}} ROH + H_2O + A \qquad (3)$$

The reaction pathways by which these enzymes act are complex, and we will have occasion later to remark on the structure of some intermediates observed during their catalytic cycles.

Considerable emphasis (arising historically from studies on the heme function in hemoglobin and the cytochromes) has been placed upon the role of the central metal ion. However, the ubiquity of metalloporphyrins in nature and the many varied functions they perform suggest that, besides modifying metal redox potentials or binding the metal at an appropriate site in the protein, the porphyrin ring itself possesses properties necessary for proper biological function. In this Account the results of porphyrin oxidations will be discussed in relation to their biological function and significance.

Oxidation of Model Porphyrins

Polarographic studies[8] have shown that a variety of porphyrins and metalloporphyrins undergo two successive one-electron oxidations. The synthetic pigments most thoroughly investigated are metallooctaethylporphyrins (**5**) (MOEP) and metallotetraphenylporphyrins (**6**) (MTPP). Porphyrins containing magnesium and zinc may be readily oxidized by iodine or bromine[9-11] in CH_2Cl_2, $CHCl_3$, and

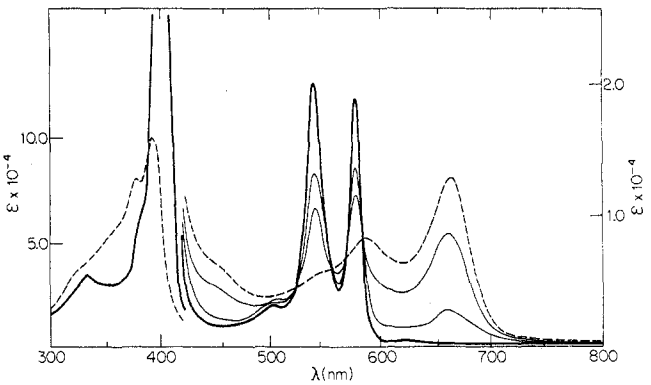
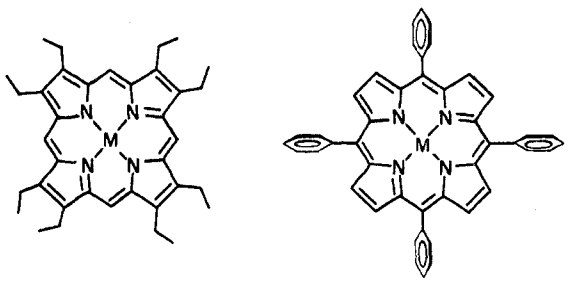


Figure 2. Optical absorption spectra of MgOEP (heavy solid line) and $[Mg^{II}OEP]\cdot^+ClO_4^-$ (dashed line) in methylene dichloride.



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CH_3OH, by electrolysis in CH_2Cl_2 or butyronitrile,[12] or by photochemical oxidation.[13] Stoichiometric titrations with chemical oxidants or coulometry during electrolysis agree with the one-electron process observed polarographically, and analyses[12,14] of the epr hyperfine structure (hfs) of the singly oxidized species $[Zn^{II}TPP]\cdot^+$, $[Mg^{II}TPP]\cdot^+$, and $[Mg^{II}OEP]\cdot^+$ have clearly shown that the radicals are formed by electron abstraction from porphyrin orbitals. However, when the epr spectra of $[Mg^{II}OEP]\cdot^+$ and $[Mg^{II}TPP]\cdot^+$ were compared (Figures 1a and 1b), striking differences were noted. Thus $[Mg^{II}TPP]\cdot^+$ shows hfs from four equivalent nitrogens and from phenyl protons, while $[Mg^{II}OEP]\cdot^+$ exhibits a five-line spectrum due to four equivalent meso protons, but shows no coupling to the nitrogens (assignments were verified by deuterium substitution (Figures 1a and 1b)). Hence the epr spectra, while establishing the oxidized products as π cation radicals, lead to the question: why should spin density distributions be different in these radicals? Moreover, one must ask why are the optical spectra (Figures 2 and 3) of these apparently similar species so different?[15]

Theoretical considerations suggest an answer to these questions. The theory of porphyrin optical spectra developed by Gouterman and coworkers[18] indicates that the highest filled orbitals, a_{1u} and a_{2u},

(8) A. Stanienda and G. Biebl, *Z. Phys. Chem. (Frankfurt am Main)*, **52**, 254 (1967); A. Stanienda, *Z. Phys. Chem. (Leipzig)*, **229**, 257 (1965).
(9) J.-H. Fuhrhop and D. Mauzerall, *J. Amer. Chem. Soc.*, **90**, 3875 (1968).
(10) R. H. Felton, D. Dolphin, D. C. Borg, and J. Fajer, *J. Amer. Chem. Soc.*, **91**, 196 (1969).
(11) J.-H. Fuhrhop and D. Mauzerall, *J. Amer. Chem. Soc.*, **91**, 4174 (1969).
(12) J. Fajer, D. C. Borg, A. Forman, D. Dolphin, and R. H. Felton, *J. Amer. Chem. Soc.*, **92**, 3451 (1970).
(13) A. N. Sidorov, V. Ye Kholmogorov, R. P. Yestigneyeva, and G. N. Kol'tsova, *Biofizika*, **12**, 143 (1968).
(14) J. Fajer, D. C. Borg, A. Forman, R. H. Felton, L. Vegh, and D. Dolphin, *Ann. N. Y. Acad. Sci.*, **206**, 349 (1973).
(15) The pronounced 660-nm absorption in $Mg^{II}OEP\cdot^+$ led to a preliminary identification[9] of this species as a metallophlorin[16,17] radical formed by nucleophilic attack at a meso carbon; however, the epr data obviate this structure.
(16) R. B. Woodward, *Ind. Chim. Belge*, **11**, 1293 (1962).
(17) D. Mauzerall, *J. Amer. Chem. Soc.*, **84**, 2437 (1962).
(18) M. P. Gouterman, *J. Mol. Spectrosc.*, **6**, 138 (1961); C. Weiss, H. Kobayashi, and M. P. Gouterman, *ibid.*, **16**, 415 (1965).

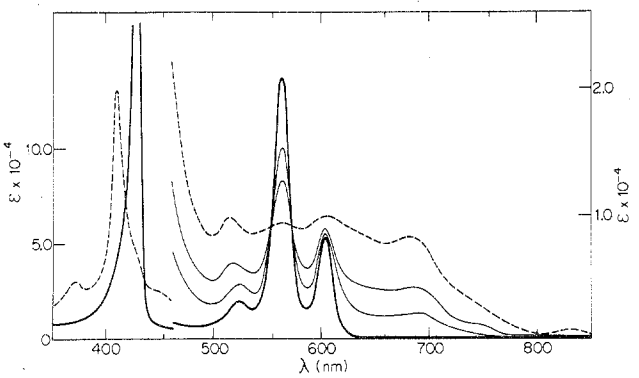


Figure 3. Optical absorption spectra of MgTPP (heavy solid line) and $[Mg^{II}TPP]\cdot^{+}ClO_4^-$ (dashed line) in methylene dichloride.

are almost degenerate in the unoxidized species. Therefore a π cation radical produced by electron abstraction from one or the other of these orbitals would lead to either a $^2A_{2u}$ or $^2A_{1u}$ ground state. This qualitative consideration is borne out by the results of open-shell calculations which predict an energy gap of 2000 to 3000 cm^{-1} between these two states.[12] In the $^2A_{2u}$ state, spin density appears on the meso carbon and nitrogen atoms, while the $^2A_{1u}$ state has low spin density on these atoms. In the latter state the spin density is primarily confined to α-pyrrolic carbon atoms. Predicted values for the nitrogen and hydrogen hyperfine coupling constants agree with those observed in $[Zn^{II}TPP]\cdot^{+}$. The small energy gap implies that peripheral substituents on the porphyrin ring, the metal, or its axial ligands might be expected to influence which ground state is lowest in energy. The epr data cited above show this to be the case; $[Mg^{II}OEP]\cdot^{+}$ has a $^2A_{1u}$ ground electronic state while $[Mg^{II}TPP]\cdot^{+}$ has the spin distribution of a $^2A_{2u}$ ground state.

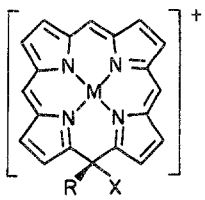
Electronic transitions in the doublet manifold will differ as one or the other state is the ground state of the system. We have used optical spectra, particularly in the region 500–700 nm, to assign ground states to the π cation radicals. Examples[11,19,20] of species with A_{1u} spectra are $[Mg^{II}OEP]\cdot^{+}ClO_4^-$ and Br^-, $[Zn^{II}OEP]\cdot^{+}ClO_4^-$ and Br^-, and $[Ni^{II}TPP]\cdot^{+}ClO_4$ and Cl^-; A_{2u} spectra are displayed *inter alia* by $[Zn^{II}TPP]\cdot^{+}ClO_4^-$ and Br^-, $[Ni^{II}OEP]\cdot^{+}ClO_4^-$, $[Cu^{II}TPP]\cdot^{+}ClO_4^-$, and $[Cu^{II}OEP]\cdot^{+}ClO_4^-$. The last two examples indicate the subtlety of which interaction or set of interactions serves to select a specific ground state. Both oxidized copper porphyrins, though differing in peripheral substituents, display spectra characteristic of electron abstraction from an a_{2u} orbital.[8]

Important evidence in favor of the two-state theory was obtained with $Co^{II}OEP$.[21] Chemical oxidation with bromine proceeded by successive reversible one-electron oxidations, *viz.*

$$Co^{II}OEP \xrightarrow{\frac{1}{2}Br_2} [Co^{III}OEP]^{+}Br^- \xrightarrow{\frac{1}{2}Br_2} [Co^{III}OEP]^{2+},2Br^- \quad (4)$$

The resulting optical spectrum was typical of a $^2A_{1u}$ state. Electrochemical preparation of the π cation radical with tetrapropylammonium perchlorate as supporting electrolyte yielded $[Co^{III}OEP]\cdot^{2+}\cdot 2ClO_4^-$, whose spectrum was that of a typical $^2A_{2u}$ ground state. When the dibromide salt was treated with a slight excess of silver perchlorate, the bromide ligands were removed and the optical spectrum changed to that of the perchlorate salt (Figure 5a). This dramatic and reversible change in spectra as the axial ligands were changed is best explained by a switching of ground states.

The further electrochemical oxidation of the π cation radicals at potentials about 0.3 V above those required for the first oxidation brought about a second reversible one-electron oxidation to give a π dication.[12] During these oxidations the epr signal of the π cation radical decreased and disappeared when the oxidation was complete, and simultaneously the optical spectra changed to give an almost featureless spectrum in the visible and a single absorption in the uv region. Whereas the π cation radicals are relatively stable species which we routinely recrystallized from nucleophilic solvents such as a methanol, the π dications are powerful electrophiles which react readily with a variety of nucleophiles such as methanol, water, acetate, and halides to give initially an isoporphyrin (7; X = original nucleophile). In the



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case of the *meso*-tetraphenyl derivatives (7; R = phenyl), these isoporphyrins are stable and have been isolated and characterized[22] as the perchlorate salts. Their optical spectra are characterized by a strong absorption band at 850–900 nm. With the octaethyl derivatives, however, the intermediate isoporphyrin (7; R = H) can lose a proton to give the neutral meso-substituted metalloporphyrin.[20] This series of reactions presents a convenient synthetic route to a variety of meso-substituted porphyrins and represents the first example of nucleophilic substitution at the periphery of the porphyrin nucleus.

The Occurrence of Metalloporphyrin π Cation Radicals in Nature

Photosynthesis. The ease of formation and stability of the metalloporphyrin π cation radicals led to the possibility that such species might occur in nature. The search began in the photosynthetic systems where chlorophyll a (Chl, 2), a magnesium dihydroporphyrin (chlorine), and bacteriochlorophyll (BChl, 3), a magnesium tetrahydroporphyrin (bacteriochlorine), play principal roles in the initial light-induced step of photosynthesis.

In the chloroplasts of plants, the majority of chlorophyll molecules have optical absorptions similar to the cell-free pigment. These function as antennae to

(19) A. Wolberg and J. Manassen, *J. Amer. Chem. Soc.*, **92**, 3982 (1970).
(20) D. Dolphin, Z. Muljiani, K. Rousseau, D. C. Borg, J. Fajer, and R. H. Felton, *Ann. N. Y. Acad. Sci.*, **206**, 177 (1973).
(21) D. Dolphin, A. Forman, D. C. Borg, J. Fajer, and R. H. Felton, *Proc. Nat. Acad. Sci. U. S.*, **68**, 614 (1971).
(22) D. Dolphin, R. H. Felton, D. C. Borg, and J. Fajer, *J. Amer. Chem. Soc.*, **92**, 743 (1970).

harvest and transport light energy to specific Chl a molecules that absorb to the red (at approximately 700 nm) of the antennae molecules. This species is known as pigment 700 (P700), and the differences in principal absorption maxima of P700 and cell-free Chl a (677 nm) arise from the local environment of P700 in the photosynthetic unit. Available evidence suggests that P700 is Chl a dimer, perhaps stabilized by a bridging water molecule.[23] A similar situation arises in photosynthetic bacterial chromatophores with BChl or chlorobium chlorophyll serving as antennae that transport energy to P865, the specialized[24] BChl a molecule in the reaction center. Upon illumination, *in vivo* bleaching of P700 or P865 and the concurrent appearance of epr signals have been interpreted as photooxidation of these species.[25-30]

The oxidation of cell-free chlorophyll a by methanolic ferricyanide produced an epr signal which mimicked those seen in photosynthesizing chloroplasts, and it was these observations that suggested that the photochemical step in photosynthesis was in fact a photooxidation of chlorophyll a. However, the site from which the electron was removed during this oxidation and the structure and fate of the remaining oxidized species have been the subjects of much speculation.

The electrochemical oxidation of chlorophyll a in methylene dichloride proceeded with the removal of one electron per molecule and gave a yellow solution (Figure 4a) which, upon electrochemical reduction, regenerated the initial chlorophyll a. Electrophoresis of this oxidized species showed it to be a cation and the radical character of the oxidized species was evident from its epr spectrum, which consisted of a single narrow line (9 G wide, peak to peak) at g = 2.0026. This epr signal closely resembled that reported for photosynthetic systems and suggested a similarity between the electrochemically oxidized chlorophyll a and the photooxidized P700 of nature. This similarity was further exemplified by the close resemblance of the difference spectra (Figure 4b) of chlorophyll a and electrochemically[31] generated Chl a·$^+$ with that of dark and photosynthesizing chloroplasts.[32] The only major difference between these two spectra was the bathochromic shift of the change at 700 nm compared to that of 677 for cell-free chlorophyll a, a shift which reflects the special environment of P700 in the photosynthetic unit. The spectral analogies between the electrochemically generated Chl a·$^+$ and photooxidized P700 strongly support

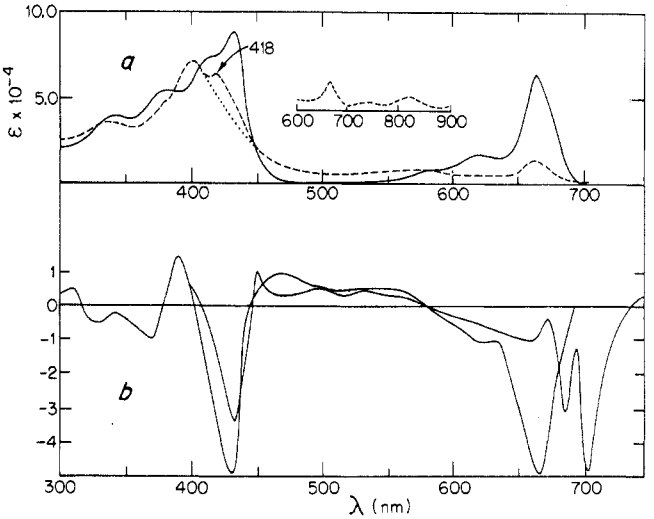

Figure 4. (a) Optical absorption spectra of Chl a (——) and Chl a·$^+$ (---) in methylene dichloride. A small amount of decomposition product (λ_{max} 418 nm) appears during the electrochemical oxidation. (b) The optical difference spectrum[32] of Chl a and Chl a·$^+$ (calculated from a) and the difference spectrum of photosynthesizing chloroplasts. The observation of an absorption band in the near infrared of the chlorophyll π cation radical (---) suggested that such an absorption should be observed in the near-infrared difference spectrum of photosynthesizing chloroplasts; such an observation[32] has been recently made.

the identification of Chl a·$^+$ as the chlorine moiety of oxidized P700.

A characterization of Chl a·$^+$ was then needed because of its role in photosynthetic processes. While there was general agreement that bleached P700 was a positive ion of chlorophyll, no evidence was adduced which demonstrated that this species was specifically a π cation radical. Fortunately this identification can be established by comparisons between Chl a·$^+$ and oxidized metalloporphyrins and chlorines.

Two reversible one-electron waves ($E_{1/2}(1)$ and $E_{1/2}(2)$) are observed in the polarographic oxidation of chlorophyll a and b, pheophytin a and b (magnesium-free chlorophylls), and zinc *meso*-tetraphenylchlorine.[8,31] The difference in half-wave potentials, $\Delta = E_{1/2}(2) - E_{1/2}(1)$, is 0.27 (±0.03) V for these chlorines. For a series of metalloporphyrins Δ = 0.30 (±0.03) V and for metalloetioporphyrins[8] Δ = 0.35 (±0.1) V. If this difference is examined rather than the individual oxidation potentials, then the influence of the metal is minimized. Moreover, Δ is constant when the sites of the two reversible one-electron abstractions are the same, and the prior unambiguous demonstration of π cation radical formation in metalloporphyrins demonstrates that chlorophyll a is similarly oxidized. Other possibilities such as oxidation of an unsaturated side chain or of the isocyclic ring of chlorophyll are ruled out by this argument and the favorable spectral comparisons between Chl a·$^+$ and the π cation radical of zinc tetraphenylchlorine.

Similar comparisons[26,33] between the properties of BChl·$^+$, produced by iodine oxidation or electrolysis, and those of bleached P865 attest that P865·$^+$ contains a π cation radical of BChl.

Since a dimer or aggregate structure for P700 or P865 is favored by epr and circular dichroism[34,35]

(23) J. R. Norris, R. A. Uphaus, H. L. Crespi, and J. J. Katz, *Proc. Nat. Acad. Sci. U. S.* **68**, 625 (1971).

(24) J. R. Norris, M. E. Dryan, and J. J. Katz, *J. Amer. Chem. Soc.*, **95**, 1680 (1973). The possibility that a moiety of the dimer is bacteriophaeophytin (Mg-free bacteriochlorophyll) is not excluded by these authors' results.

(25) D. H. Kohl, in "Biological Applications of Electron Spin Resonance," H. M. Swartz, J. R. Bolton, and D. C. Borg, Ed., Wiley-Interscience, New York, N. Y., 1972, p 213.

(26) J. D. McElroy, G. Feher, and D. Mauzerall, *Biochim. Biophys. Acta*, **267**, 363 (1972).

(27) W. W. Parson, *Biochim. Biophys. Acta*, **153**, 248 (1968).

(28) P. A. Loach and K. Walsh, *Biochemistry*, **8**, 1908 (1969).

(29) J. R. Bolton, R. K. Clayton, and D. W. Reed, *Photochem. Photobiol.*, **9**, 209 (1969).

(30) J. T. Warden and J. R. Bolton, *J. Amer. Chem. Soc.*, **94**, 4351 (1972).

(31) D. C. Borg, J. Fajer, R. H. Felton, and D. Dolphin, *Proc. Nat. Acad. Sci. U. S.*, **67**, 813 (1970).

(32) T. Hiyama and B. Ke, *Biochim. Biophys. Acta*, **267**, 160 (1972).

(33) P. A. Loach, R. A. Bambara, and F. J. Ryan, *Photochem. Photobiol.*, **13**, 247 (1971).

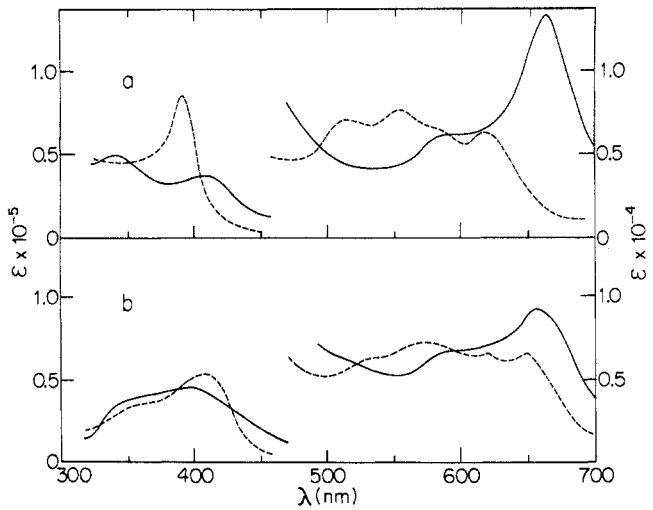

Figure 5. Comparison of the optical absorption spectra of (a) $[Co^{III}OEP]^{\cdot 2+}2Br^-$ (——) and $[Co^{III}OEP]^{\cdot 2+}2ClO_4^-$ (---); (b) Cat I (——) and HRP I (---). Catalase is tetrameric and the ε above is per hemin.

measurements, it is natural to inquire if the Chl a or BChl moiety might serve as the primary acceptor for the photochemically ejected electron and yield the anion radical of Chl or BChl, *e.g.*

$$(BChl)_2 \xrightarrow{h\nu} BChl^{\cdot +} + BChl^{\cdot -} \qquad (5)$$

Vacuum electrolytic reduction of BChl dissolved in dimethylformamide affords a facile route to the anion radical; comparison[36] of its optical absorption spectrum (maxima at 990 and 920 nm) and epr spectrum (g = 2.0028) with the spectra of the presumed primary acceptor[37-39] (no absorption in the ir and g = 2.005) distinguishes between these species. However, the photochemical reaction leading to charge separation might involve $BChl^{\cdot -}$ as a transient intermediate, observable, perhaps, by near-ir flash spectroscopic studies of reaction center preparations.

Catalase and Peroxidase. When nature uses chlorophyll as a source of electrons it is not too surprising that it is the dihydroporphyrin ring rather than the divalent magnesium ion which supplies them. With iron porphyrins, however, it has generally been assumed that the iron atom itself is the entity which undergoes the redox reaction, and in the cytochromes, which function *via* an Fe(II) ⇌ Fe(III) couple, there is no doubt that it is the metal which is the *eventual* site of electron capture or release.

There are two closely related series of iron porphyrin containing enzymes, the catalases[40] (Cat) and the peroxidases[41] (which are typified by horseradish peroxidase (HRP)). The resting enzymes both contain trivalent iron and are oxidized by hydrogen peroxide. The first intermediate observed spectrophotometrically during this oxidation is the so-called primary compound (Cat I or HRP I) which has two electrons less than the parent ferrihemoprotein. A one-electron reduction of the green primary compound forms the brown-red secondary compound (Cat II or HRP II). While the first step in the catalytic cycle of these two enzymes is the same, *i.e.*, a two-electron oxidation, by hydrogen peroxide, to their primary compounds, the two enzymes then perform different functions. Cat I oxidizes a second molecule of hydrogen peroxide to molecular oxygen and is itself reduced back to the ferrihemoprotein, while HRP I reacts with a hydrogen donor AH_2 to give a free radical and the secondary compound of the enzyme HRP II (eq 6) which can in turn oxidize a second donor molecule with the formation of the ferrihemoprotein (eq 7).

$$HRP\ I + AH_2 \longrightarrow HRP\ II + AH\cdot + H^+ \qquad (6)$$

$$HRP\ II + AH_2 \longrightarrow HRP + AH\cdot + H^+ \qquad (7)$$

There are a number of ways in which one might describe the electronic distribution in the primary complexes. It was originally suggested[42] that the primary compounds were complexes between hydrogen peroxide and the ferric iron, *i.e.*, $Fe^{III}OOH$, but this concept was challenged[40,43] when it was found that nonperoxidatic oxidants could give rise to the primary compounds. It was then suggested that the two electrons could be removed from the ferric iron to give pentavalent iron,[44] or by some combination of oxidations of the iron, the porphyrin, or the protein.[45-47] But such proposals were considered unlikely since an oxidation of either the porphyrin ring or the protein would give rise to free radicals which were considered to be too unstable and could not account for the stability exhibited by the primary complexes. To the contrary, it is our contention that the primary complexes of both catalase and horseradish peroxidase are porphyrin π cation radicals. We saw earlier that the π cation radical $[CO^{III}OEP]^{\cdot 2+}$ could, as a function of its axial ligands, be made to occupy either of two ground states. Compare now the optical spectra of these two ground states with the optical spectra of the primary compounds of catalase and horseradish peroxidase (Figure 5). The similarity strongly suggests that Cat I is a π cation radical which exists in a $^2A_{1u}$ ground state and that HRP I is likewise a π cation radical with a $^2A_{2u}$ ground state.

If porphyrin ring oxidation accounts for one oxidizing equivalent, then from where in the primary complexes is the other electron removed? A further oxidation of the ring would result in a π dication which can be excluded since its optical spectrum[12] is so different from that of Cat I or HRP I. Oxidation of the protein would result in the formation of an organic free radical which should be detectable by epr, but so far has not been observed. A suggested ferric

(34) E. A. Dratz, A. J. Schultz, and K. Sauer, *Brookhaven Symp. Biol.*, **19**, 303 (1967).
(35) K. Sauer, in "Methods in Enzymology," Vol. 24B, A. San Pietro, Ed., Academic Press, New York, N. Y., 1972.
(36) J. Fajer, D. C. Borg, A. Forman, D. Dolphin, and R. H. Felton, *J. Amer. Chem. Soc.*, **95**, 2739 (1973).
(37) P. A. Loach and R. L. Hall, *Proc. Nat. Acad. Sci. U. S.*, **68**, 1010 (1971).
(38) R. K. Clayton, *Proc. Nat. Acad. Sci. U. S.*, **69**, 44 (1972).
(39) G. Feher, M. Y. Okamura, and J. D. McElroy, *Biochim. Biophys. Acta*, **267**, 222 (1972).
(40) P. Nicholls and G. R. Schonbaum, in "The Enzymes," 2nd ed., P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, New York, N. Y. 1963, Chapter 6.
(41) K. G. Paul, in "The Enzymes," 2nd ed., P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press New York, N. Y., 1963, Chapter 7.
(42) B. Chance, *Nature (London)*, **161**, 914 (1948).
(43) P. George, *J. Biol. Chem.*, **201**, 413 (1953).
(44) M. E. Winfield, in "Haematin Enzymes," J. E. Falk, R. Lemberg, and R. K. Morton, Ed., Pergamon Press, Elmsford, N. Y., 1961, p 245.
(45) A. S. Brill and R. J. P. Williams, *Biochem. J.*, **78**, 253 (1961).
(46) J. Peisach, W. E. Blumberg, B. A. Wittenberg, and J. B. Wittenberg, *J. Biol. Chem.*, **243**, 1871 (1968).
(47) A. S. Brill, *Compre. Biochem.*, **14**, 447 (1966).

isoporphyrin structure[48] also may be excluded, since authentic species have recently been prepared;[49] their electronic absorption spectra show characteristic bands at 870–900 nm ($\epsilon \sim 14{,}000$) which are absent in Cat I or HRP I.

These considerations require that the oxidation of the ferrihemoproteins be accounted for by the loss of one electron from the metal (to give Fe(IV)) and one from the ring (to give a π cation radical). The overall electronic structure of the primary complexes can then be represented as

$$[Fe^{IV}HRP]^{3+} \text{ and } [Fe^{IV}Cat]^{3+} \quad (8)$$

The secondary compounds, prepared by a one-electron reduction of the primary compounds, display an optical spectrum typical of a metalloporphyrin. This suggests that the electron adds to the ring and thus the secondary compounds are derivatives of tetravalent iron porphyrins, a view consonant with the original hypothesis of George.[43] Supporting evidence comes from Mossbauer spectroscopy[50,51] which demonstrates that the electronic structure of iron in the primary and secondary compounds is the same but differs from that found in the parent ferrihemoprotein.

The existence of tetravalent iron in metalloporphyrins is controversial, but the preparation and characterization[52,53] of singly oxidized ferric porphyrins support this view. Electrochemical oxidation with simultaneous coulometry of $Fe^{III}TPP^{+}Cl^{-}$, $Fe^{III}OEP^{+}Cl^{-}$, and the μ-oxo dimers, $(Fe^{III}TPP)_2O$ and $(Fe^{III}OEP)_2O$, afforded stable products. Proton magnetic resonance spectra of the oxidized porphyrins were interpreted as evidence against a π cation radical formulation, since both the magnitudes of the paramagnetic shifts and the line widths are inconsistent with appreciable spin density at the porphyrin periphery. This conclusion is supported by extended Hückel calculations[54] of the high-spin iron monomer which predict that the highest filled MO is composed of approximately equal amounts of $d_{x^2-y^2}$ and nitrogen σ orbitals. Similarly, μ-oxo dimer orbitals likely to be involved in the oxidation contain negligible contributions from porphyrin π atomic orbitals. The observation of an integer spin ($S = 2$) in $[Fe^{IV}TPP]^{2+}$ and $[Fe^{IV}OEP]^{2+}$ agrees well with a similar result in the two-electron oxidation of cytochrome c peroxidase (with ethyl hydrogen peroxide), where Mossbauer spectra suggest that the iron in the oxidized enzymes is characterized by an integer spin.[55]

It is interesting to speculate as to why these two similar enzymes function so differently, for while catalase decomposes hydrogen peroxide by a diffusion-controlled rate, horseradish peroxidase shows no catalytic activity of this type and is stable in the presence of excess hydrogen peroxide.[40] Moreover, while HRP I reacts with hydrogen donor substrates to give free radicals,[41] catalase decomposes hydrogen peroxide by a nonradical pathway.[56] We suggest that the two ground states and the different electronic distributions associated with them account for this difference in reactivity.

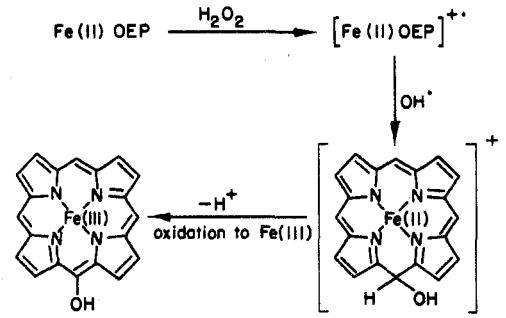

Figure 6. Mechanism proposed to account for the oxidation of ferrous *octaethylporphyrin* to ferric *meso*-hydroxyoctaethylporphyrin.

Cytochromes and Heme Catabolism. The catabolism of heme to bile pigments proceeds through the intermediacy of *meso*-hydroxyhemin.[57] Ferric *meso*-hydroxyoctaethylporphyrin was formed when ferrous, but not ferric, octaethylporphyrin was treated with hydrogen peroxide in pyridine.[58] This unusual observation was explained by assuming an analogy to Fenton's reagent, where ferrous iron catalyzes the conversion of hydrogen peroxide to a hydroxyl radical. By analogy it was assumed that the $Fe^{II}OEP$ and hydrogen peroxide would react to give $Fe^{III}OEP$ and an hydroxyl radical, and that these would react in turn to give ferric *meso*-hydroxyoctaethylporphyrin. But meso substitution of $Fe^{III}OEP$ with either hydroxyl or benzoyloxy radicals does not occur even though metalloporphyrins such as Zn- and MgOEP react very rapidly with benzoyl peroxide to give *meso*-benzoyloxyporphyrins.[20] The mechanism by which the last reaction proceeds may, however, throw some light on the meso hydroxylation of $Fe^{II}OEP$.

When $Mg^{II}TPP$ is treated with a slight excess of benzoyl peroxide it is oxidized to the corresponding π cation radical $[Mg^{II}TPP]^{\cdot+}$, and in the presence of excess benzoyl peroxide this π cation radical reacts to give *meso*-benzoyloxymagnesium tetraphenylisoporphyrin. When the same reaction is carried out with $Mg^{II}OEP$, the π cation radical is once again formed, and this reacts to give the *meso*-benzoyloxyisoporphyrin, which reacts further to give the corresponding meso-substituted MgOEP. If one applies this same mechanism to the meso hydroxylation of $Fe^{II}OEP$, the steps could be represented as follows (Figure 6). This mechanism requires that the one-electron oxidation of a ferrous porphyrin initially generates the corresponding ferrous porphyrin π cation radical and not the ferric porphyrin, and such an

(48) D. H. Busch, K. Farmery, V. Goedken, V. Katovic, A. C. Melnyk, C. R. Sperati, and N. Tokel, *Advan. Chem. Ser.*, **100**, 44 (1971).
(49) J. A. Guzinski and R. H. Felton, *J. Chem. Soc., Chem. Commun.*, 714 (1973).
(50) T. H. Moss, A. Ehrenberg, and A. J. Bearden, *Biochemistry*, **8**, 4159 (1969).
(51) Y. Maeda and Y. Morita, in "Structure and Function of Cytochromes," K. Okunuki, M. D. Kamen, and I. Sekuzu, Ed., University of Tokyo Press, 1968, p 523.
(52) R. H. Felton, G. S. Owen, D. Dolphin, A. Forman, D. Borg, and J. Fajer, *Ann. N. Y. Acad. Sci.*, **206**, 504 (1973).
(53) R. H. Felton, G. S. Owen, D. Dolphin, and J. Fajer, *J. Amer. Chem. Soc.* **93**, 6332 (1971).
(54) M. Zerner, M. P. Gouterman, and H. Kobayashi, *Theor. Chim. Acta.*, **6**, 363 (1966).
(55) G. Lang, *Quart. Rev. Biophys.*, **3**, 1 (1970).
(56) R. K. Bonnichsen, B. Chance, and H. Theorell, *Acta Chem. Scand.*, **1**, 685 (1947).
(57) C. O'Heocha, in "Porphyrins and Related Compounds," T. W. Goodwin, Ed., Academic Press, New York, N. Y., 1968.
(58) R. Bonnett and M. J. Dimsdale, *Tetrahedron Lett.*, 731 (1968); R. Bonnett and M. J. Dimsdale, *J. Chem. Soc.*, 2540 (1972).

hypothesis explains the lack of reaction of the ferric porphyrin toward hydrogen peroxide.

If the oxidation of the ferrous porphyrin can give a ferrous porphyrin π cation radical, then this offers an attractive explanation to account for the redox properties of the cytochromes, such as cytochrome c. Crystallographic structure determinations have shown that the iron atoms of cytochrome c[59] and cytochrome b_5[60] are so well "protected" by the porphyrin and protein that *in vivo* electron carriers cannot approach the iron directly. How then do the cytochromes function when the iron atom is so inaccessible? Suggestions have been advanced[61,62] that the initial attack in the electron-transfer process occurs at the porphyrin, a segment of which is exposed to the exterior of the protein sheath. Complete removal of an electron would give the ferrocytochrome π cation. Rapid internal transfer could then occur between the ring and metal to give the ferrihemoprotein.

$$Fe^{II}Cyt\ c \xrightarrow{-e^-} [Fe^{II}Cyt\ c]^{\cdot+} \xrightarrow[\text{transfer}]{\text{internal}} Fe^{III}Cyt\ c \quad (9)$$

Analogies are known for such internal transfers. Thus the one-electron oxidation of $Pb^{II}OEP$ gives $[Pb^{II}OEP]^{\cdot+}$, and the second one-electron oxidation of this species gives $Pb^{IV}OEP$.[63,64] One can envisage this second oxidation as an oxidation of the metal or of the ring, but in either case an internal redistribution of electrons must take place to give the tetravalent lead porphyrin. Similar internal conversion of this type has been observed in cobalt complexes of porphyrin-like macrocycles[48,65] and during the oxidation of $Pd^{II}TPP$ to $Pd^{IV}TPP$.[66]

$$Pb^{II}OEP \xrightarrow{-e^-} \begin{array}{ccc} & \xrightarrow{-e^-} & [Pb^{III}OEP]^{+} \\ [Pb^{II}OEP]^{\cdot+} & & \downarrow \text{internal transfer} \quad Pb^{IV}OEP \\ & \xrightarrow{-e^-} & [Pb^{II}OEP]^{2+} \end{array}$$

One may ask if systems such as a ferrous π cation radical and a ferric porphyrin (eq 9) differ only in the way in which their electronic structures are written are they not simply both resonance forms of the same molecule, and if so why consider them as two distinct species? It is clear, however, in the cases of the cobalt and lead species described above, that while the two partners in the reaction are isoelectronic there is apparently an energy barrier between their interconversion. We envisage that such a barrier may be the result of either a conformation change or change in ligation of the metal atom occurring during the internal transfer.

The role of ferrocytochrome π cation in the electron-transfer mechanism is speculative; indeed, reaction of ferricytochrome with electrons or hydrogen atoms produced by pulse radiolysis has been interpreted[67,68] as evidence that transfer occurs *via* the protein residues.[69] However, the mechanism indicated in eq 9 might be tested by pulse radiolysis or flash photolysis of the protein-free heme. At present, no mechanistic assignment can be made with surety.

Conclusion

Metalloporphyrins, chlorines, and bacteriochlorines undergo reversible one-electron oxidations to give π cations. It has been shown by spectral comparisons that the *in vivo* bleaching of the photosynthetic systems P700 and P865 corresponds to the formation of a π cation radical of chlorophyll or bacteriochlorophyll. Moreover, the spectral properties of the enzymatically active primary complexes of both catalase and horseradish peroxidase suggest that they are π cation radicals containing tetravalent iron, a characterization which is supported by the preparation of authentic iron(IV) porphyrins. The formation of π cation radicals when zinc or magnesium porphyrins are treated with organic peroxides, as well as the difference in reactivity of the ferrous and ferric complexes under these conditions, leads to the hypothesis that electron transport involving the cytochromes takes place *via* ring oxidation followed by rapid internal conversion.

Attention has begun to focus on the influence of axial ligands on the course as well as the rate[70] of electron transfer. The possibility of these acting to switch the electron transfer from metal to ring is realized in ruthenium complexes[71] and is likely important in hematin enzyme function.

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(59) T. Takano, R. Swanson, O. B. Kallai, and R. E. Dickerson, *Symp. Quant. Biol.*, 397 (1971).
(60) F. S. Mathews, M. Levine, and P. Argos, *J. Mol. Biol.*, **64**, 449 (1972).
(61) C. E. Castro, *J. Theor. Biol.*, **33**, 475 (1971).
(62) N. Sutin and A. Forman, *J. Amer. Chem. Soc.*, **93**, 5274 (1971); N. Sutin, *Chem Brit.*, **8**, 148 (1972).
(63) D. G. Whitten, T. J. Meyer, F. R. Hopf, J. A. Ferguson, and G. Brown, *Ann. N. Y. Acad. Sci.*, **206**, 516 (1973).
(64) M. P. Gouterman, P. Smith, and D. Dolphin, unpublished results.
(65) N. S. Hush and I. S. Woolsey, *J. Amer. Chem. Soc.*, **94**, 4107 (1972).
(66) L. Vegh and D. Dolphin, unpublished results.
(67) N. N. Lichtin, A. Shafferman, and G. Stein, *Science*, **179**, 680 (1973).
(68) N. Lichtin, J. Ogdan, and G. Stein, *Isr. J. Chem.*, **9**, 579 (1971).
(69) R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, and L. Samson, "Proceedings of the Wenner-Gren Symposium on Oxidative Enzymes, Aug 1970," Wenner-Gren Foundation, Stockholm, Sweden, 1971.
(70) K. M. Kadish and D. G. Davis, *Ann. N. Y. Acad. Sci.*, **206**, 495 (1973).
(71) G. M. Brown, F. R. Hopf, J. A. Ferguson, T. J. Meyer, and D. G. Whitten, *J. Amer. Chem. Soc.*, **95**, 5939 (1973).