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The \( \pi \)-Cation Radical of Chlorophyll \( \alpha \)*

Donald C. Borg, Jack Fajer, Ronald H. Felton, and David Dolphin

Abstract. Chlorophyll \( \alpha \) undergoes reversible one-electron oxidation in dichloromethane and butyronitrile. Removal of the electron by controlled potential electrolysis or by stoichiometric charge transfer to a known cation radical yields a radical (epr line width = 9 gauss, \( g = 2.0025 \pm 0.0001 \)) whose optical spectrum is bleached relative to that of chlorophyll. Upon electrophoresis this bleached species behaves as a cation. By comparison with the known properties of \( \pi \)-cation radicals of porphyrins and chlorins, the chlorophyll radical is also identified as a \( \pi \)-cation. Further correlation of optical and epr properties with published studies on photosynthesis leads to the conclusion that oxidized P700, the first photochemical product of photosystem I in green plants, contains a \( \pi \)-cation radical of the chlorin component of chlorophyll \( \alpha \). This radical is the likely source of the rapidly-decaying, narrow epr signal of photosynthesis.

Some years ago it was proposed that the narrow, rapidly-decaying epr signal of photosynthesis (Signal I of Commoner)\(^5\) might be due to removal of an electron from chlorophyll (Chl) or bacteriochlorophyll, or from closely related pigments.\(^3\)-\(^6\) Despite various objections,\(^7\)-\(^10\) it is now widely conceded that Signal I represents the first chemical product of system I, and results from photooxidation of a photoconverter chlorophyll pigment, P700, or from bacteriochlorophyll in a specialized environment (PS70) in the case of bacteria.\(^11\)-\(^19\)

It is established that oxidation of Chl by ferricyanide produces optical changes (bleaching of the \( \sim 700 \) nm absorption band) and epr signals (\( g = 2.0025 \pm 0.0001 \), width 7-13 gauss, gaussian line shape, absence of hyperfine structure) like those caused by light. In fact, this complementarity identifies the products of ferricyanide oxidation as the same Chl species as are involved in the quantum conversion steps of photosynthesis.\(^5\),\(^8\),\(^13\),\(^20\)

Addition of ferric chloride to methanolic solutions of chlorophyll \( \alpha \) bleaches the strong absorptions of Chl.\(^21\) Immediate addition of a mild reductant, such as cuprous chloride, regenerates Chl from the yellow solution.\(^22\) A green color also returns to the solution if it is left standing or if water is added, but allomers, as well as chlorophyll \( \alpha \), are then present.

Rabinowitch and Weiss\(^21\) suggested three possible formulations of the bleached species produced by ferric chloride oxidation:

\( A \) electron abstraction (site unspecified) to form a positive radical (Chl\( ^{+} \)) of chlorophyll \( \alpha \),
(B) subsequent attack on the Chl$^+$ by a nucleophile to form a neutral radical (the nucleophile might be chloride or methanol\textsuperscript{23}), and

(C) irreversible loss of a proton from Chl$^+\cdot$. They favored reaction A, as did Linschitz and Rennert, who prepared a bleached product by photooxidation in low-temperature glasses.\textsuperscript{24} The feature distinguishing Chl$^+\cdot$ from the remaining possibilities is the positive charge, and preliminary moving boundary experiments indicated possible formation of Chl$^+\cdot$.\textsuperscript{21} Later Stanienda demonstrated\textsuperscript{25} by cyclic voltammetry, two successive oxidations at a platinum electrode:

\[
\text{Chl} \rightarrow \text{Chl}^+\cdot + e^- \quad E_{\text{f0}} = 0.52 \text{ V},
\]

\[
\text{Chl}^+\cdot \rightarrow \text{Chl}^{2+} + e^- \quad E_{\text{f1}} = 0.77 \text{ V [versus sce (aq)]}.
\]

However, bulk electrolysis in propionitrile yielded spectra which differed from that of the bleached product from ferric chloride. Diehn and Seely subsequently revived formulation (B) and suggested\textsuperscript{23} that the bleached species is formed by solvent attack upon Chl$^+\cdot$.

We recently reported\textsuperscript{26,27} the existence of stable π-cation radicals and π-radicals of several metalloporphyrins (i.e., electrons have been removed from the organic π system), prepared by electrolysis in aprotic solvents. The relevance of these findings to the Chl oxidation prompted us to reinvestigate the nature and formation of bleached chlorophyll a. In a preliminary communication, we demonstrated\textsuperscript{26} that controlled-potential, one-electron oxidation of ethyl chlorophyllide a yielded a radical whose optical spectrum resembles that of the bleached species produced from chlorophyll a by oxidation with ferric chloride\textsuperscript{21-23} or ferric perchlorate.\textsuperscript{28}

In fact, the absorbance changes produced\textsuperscript{26} by univalent oxidation of ethyl chlorophyllide a closely resemble the difference spectra reported for light-exposed chloroplast preparations.\textsuperscript{29-31} The work reported here extends these findings to chlorophyll a, and the results demonstrate that the bleached species is, specifically, a π-cation radical of chlorophyll.

**Methods.** The techniques used for controlled potential electrolysis, coulometry, optical, and epr measurements, as well as for purification and preparation of materials, have been previously described.\textsuperscript{27} For charge-transfer reactions, a quartz epr tube was part of an ampoule with two side arms, one of which contained zinc tetrabenzyloporphyrin perchlorate (ZnPh$_4$P$^+$ClO$_4^-$) and the other Chl. Dry solvent was distilled into the tube on a vacuum line. After the cell was sealed, the dry contents of one or both side arms could be dissolved. Relative concentrations of free radicals were determined by double integration of spectra by computer under the same experimental conditions.

The electrophoresis experiment allowed rapid migration of the putative Chl$^+$ (without carrier electrolyte) in order to minimize decomposition. Chl$^+$ was prepared by the charge-transfer reaction:

\[
\text{ZnPh}_4\text{P}^++ + \text{Chl} \rightarrow \text{ZnPh}_4\text{P}^0 + \text{Chl}^++ \quad (1)
\]

since the stable ZnPh$_4$P$^+$ cation radical has a higher potential [$E_{\text{f0}}(1) = +0.71 \text{ V}$] (ref. 27) than that required for oxidation of Chl a [$E_{\text{f0}}(1) = +0.52 \text{ V}$]. The electrophoretic apparatus consisted of a U-shaped tube (3 mm i.d.) with arms 6 cm long. The lower portion of the tube was filled with Chl in deaerated CH$_2$Cl$_2$ (d = 1.31 g/cm$^3$ at 25°C), ZnPh$_4$P$^+$ClO$_4^-$ was introduced into the arm which would become the anodic side, and both arms were filled with deaerated dimethylformamide (d = 0.94 g/cm$^3$ at 25°C) until
the platinum electrodes at the top of the U were submerged. This procedure established a boundary and avoided any breakdown of CH₂Cl₂ at the electrodes at the high potentials used (250–700 V). A quartz cell of 1 mm path length in the cathodic arm of the U allowed spectrophotometric measurements. For epr monitoring, a U-tube was filled with deaerated CH₂Cl₂, and an aliquot of a deaerated CH₂Cl₂ solution of Chl, partially titrated with ZnPh₄P⁺ ClO₄⁻, was added to the anodic limb and the arms capped with dimethyl formamide. After 250 V had been applied, small samples were removed serially from the cathodic limb for epr examination.

Results. Electrochemical formation of a chlorophyll free radical: At a potential of 0.60 V (versus sce), chlorophyll a in CH₂Cl₂ underwent a one-electron oxidation (1.0 ± 0.1 electron/molecule) to yield a yellow solution. The product had the optical spectrum shown in Fig. 1 and did not display strong visible fluorescence. The reaction was reversible, as shown by regeneration of up to 90% of the initial Chl a upon electroreduction (E₁/₂ = 0.52 ± 0.02 V) of the bleached species. The absorption spectrum of the recovered material was that of Chl a rather than of allomerized products,²⁸ and re-electrolysis at 0.60 V regenerated the initial bleached species.

Epr singlets of gaussian shape and 9 G linewidth (peak-to-peak) were recorded at g = 2.0025 ± 0.0001 following electrolysis in situ in dichloromethane or butyronitrile (Fig. 2a). Modulation amplitudes down to 0.05 G with applied microwave power down to 2.6 mW and temperatures from −110°C to 23°C failed to resolve hyperfine structure. The signals decayed slowly when the electrolysis circuit was opened, and they disappeared rapidly with electroreduction.

Notwithstanding various purification and drying procedures applied to CH₂Cl₂ and Chl a, a slow (2–10% per 15 min) decomposition of the bleached species was observed. One product of the decomposition was identified by its spectrum as Chl a. Since about 25 min were required for electrooxidation of Chl, some decay had occurred before the spectrum of Fig. 1 was recorded. The peak at 418 nm grew in time at the expense of bleached Chl and therefore is assigned to a decomposition product. After electroreduction of the bleached solution back to Chl, the spectral difference between the initial and recovered Chl represents the absorption spectrum of the decomposition product. That absorption is subtracted in the corrected curve of Fig. 1.

Charge-transfer formation of a chlorophyll free radical: Epr experiments demonstrated stoichiometric charge transfer from ZnPh₄P⁺⁺ to Chl. After

![Fig. 1. Absorption spectra of chlorophyll a in CH₂Cl₂ (—), and its one-electron oxidation product (---), corrected for decomposition product (···).](image-url)
Fig. 2. (a) Epr spectra of the one-electron oxidation products of chlorophyll a and ethyl chlorophyllide a (lighter tracing) in butyronitrile. Electrolysis in situ at −50°C Mod. ampl. 1 G.

(b) Epr spectra of ZnPh₄P⁺⁺ClO₄⁻ in CH₂Cl₂ titrated with chlorophyll a at 22°C. (1): ZnPh₄P⁺⁺; (2) ZnPh₄P⁺⁺ and Chl⁺⁺ present (about 3:2); (3) Chl⁺⁺. Mod. ampl. 0.5 G.

ZnPh₄P⁺⁺ClO₄⁻ in CH₂Cl₂ was transferred into the side arm of a quartz ampoule containing an excess of dry Chl and then returned to the epr observation tube, the 9-line epr spectrum of ZnPh₄P⁺⁺ (ref. 27) was completely replaced by the characteristic singlet of Chl⁺⁺ (Fig. 2b).

With an excess of Chl, quantitation of free radicals by double integration of the first derivative spectra showed complete conversion of ZnPh₄P⁺⁺ to Chl⁺⁺. Stoichiometric (1:1) charge transfer was obtained in toluene as well as in dichloromethane. In the former, Chl⁺⁺ signal decay appeared to be second order (giving a linear regression with the reciprocal of signal amplitude plotted against time) for about 1 hr, while a more concentrated solution of Chl⁺⁺ in CH₂Cl₂ followed second-order decay kinetics for about 20 min. The characteristic infrared bands of the chlorophyll radical that are not obscured by ZnPh₄P were also observed after charge transfer.

**Electrophoresis to establish the ionic nature of the chlorophyll free radical:**
The radical formed as in Eq. (1) was sufficiently stable to permit its electromigration as a cation. When a potential of 250 V was applied to the U-shaped
electrophoresis cell, a yellow color was observed to migrate toward the cathode; reversing the sign of the potential reversed the direction of migration. The optical spectrum of the moving species was that of bleached chlorophyll $a$.

Epr monitoring of the electrophoresis detected the cathodic migration of a species whose spectrum was identical with that of the oxidation product formed electrolytically or by charge transfer from ZnPh$_4$P$^{+}$. As a check that the epr signal reflected electrophoresis of a charged species rather than bulk movement of solution, optical spectra were taken after the complete decay of the epr signal. These revealed absorptions due to Chl and allomerized material, as expected, while the contamination due to ZnPh$_4$P was $<2\%$.

**Formation of a free radical from a known chlorin:** Zinc tetraphenylechlorin contains no unsaturated side chains or other easily oxidizable substituents. Electrolysis of this compound at 0.79 V resulted in a yellow solution characterized by both an unstructured epr signal and the optical spectrum shown in Fig. 3. The spectrum of the stable one-electron product bears strong similarity to the spectra of the bleached species of ethyl chlorophyllide $a$ and of chlorophyll $a$; i.e., weak absorption in the near infrared and visible, and a more intense peak in the ultraviolet.

**Discussion.** The results are consistent with identification of bleached chlorophyll $a$ as a cation radical (formulation $A$). The diverse modes of preparation of the bleached species: ($a$) ferric chloride oxidation, ($b$) electrolytic oxidation, and ($c$) charge transfer with ZnPh$_4$P$^{+}$, agree with Chl$^+$ as a product; and the positive charge associated with the oxidation product rules out reactions $B$ and $C$. Polarographic data not only substantiate formulation $A$, but in addition yield information as to the site of electron abstraction. The quantity $\Delta = E_{1/2}(2) - E_{1/2}(1)$ represents the difference in half-wave oxidation potentials for the first and second electrode processes and ranges from 0.30 (±0.03) V for tetraphenylporphyrins to 0.35 (±0.1) V for etioporphyrins and 0.27 (±0.03) V for the chlorins Chl $a$ and $b$, pheophytin $a$ and $b$, and zinc tetraphenylechlorin. If this difference is examined, rather than the individual potentials, the influence of the metal substituent is minimized. It is $\Delta$ which is constant if the sites of electron

![Figure 3](image-url)
abstraction are the same. Since we have unambiguously established,26,27 via epr and optical spectra, that zinc and magnesium porphyrins are oxidized to yield \( \pi \)-cation radicals, we conclude that Chl \( a \) is similarly oxidized. Other possibilities such as specific oxidation of a substituent group of Chl \( a \) are ruled out by the above argument and by the close similarity of the electronic spectra of Chl\( +^\cdot \) and ZnPh\( 4P^+^\cdot \). The latter compound contains no substituents that are oxidized in the potential range 0.6–0.8 V.

A possible formulation for the bleached species not considered by previous authors is dimer formation

\[
(D) \quad 2\text{Chl}^+^\cdot = (\text{Chl}^+^\cdot)_2.
\]

However, the epr signal of the bleached species would require that the postulated dimer be weakly coupled, and previous work has demonstrated that the dimer of the magnesium octaethylporphyrin cation radical did not display an epr signal.27 It is unlikely that reaction \( D \) proceeds to an appreciable extent.

The instability of Chl\( +^\cdot \) in methanol or CH\( \text{H}_2\text{C}_2 \) may be due to a disproportionation reaction followed by nucleophilic attack on the dication so formed, namely,

\[
2\text{Chl}^+^\cdot \rightleftharpoons \text{Chl} + \text{Chl}^2^+ \tag{2}
\]

followed by

\[
\text{Chl}^2^+ + \text{HOH} \rightarrow \text{Chl(OH)}^+, \tag{3}
\]

and

\[
\text{Chl(OH)}^+ \rightarrow \text{allomers}. \tag{4}
\]

These reactions34 explain the partial regeneration of chlorophyll \( a \) in the absence of reducing agents. The dismutation of Eq. (2) is compatible with the second-order decay kinetics followed initially by the Chl\( +^\cdot \) epr signals in the charge-transfer experiments. A reaction analogous to Eq. (3) has been observed32 with magnesium or zinc tetraphenylporphyrins, namely, ZnPh\( 4P^2^+ + \text{CH}_3\text{OH} \rightarrow \text{ZnPh}_4\text{P(OCH}_3)_2^+ + \text{H}^+. \)

Also, under the conditions encountered in our experiments (and in methanol38), attempts to oxidize Chl\( +^\cdot \) to the dication led only to allomerized material.

Beinert and Kok inferred19 that the "fast" light-induced epr signal associated with photosynthesis was not composed of both photooxidant and photoreductant but was a single spectrum due to only one free radical species. The epr spectrum we have recorded from Chl\( +^\cdot \) closely resembles those reported from photosynthetic systems: gaussian singlets with g-values = 2.0025 and line widths of 7–13 G.1–2,5,9,12,15,16,36,27 Yet, we can be sure that in our experiments only the Chl\( +^\cdot \) cation is a signal-source. Thus we can support the conclusion of Beinert and Kok.19

Furthermore, the effect of deuteration upon the epr signal of photosynthesis is consistent with a \( \pi \)-radical with linewidth contributions from both hydrogens and nitrogens. Thus, the light-induced epr signal from deuterated (and hydrated) Chl \( a \) is narrower than that of native Chl \( a \),35 while the signals from green chlorella4 and purple photosynthetic bacteria4,15 are narrowed by 2–3 times after deuterium substitution. This was interpreted9,15 as due to magnetic interactions with hydrogens, and is consistent with delocalization into a \( \pi \)-system.35
Kohl et al. concluded\(^*\) that there was little delocalization of the unpaired electron of Signal I onto the four nitrogen atoms of Chl. McElroy and colleagues deduced nitrogen splittings of about 1 G in bacteriochlorophyll.\(^{18}\) In the case of closely related porphyrin cation radicals we showed that epr spectra may be interpreted in terms of a dominant nitrogen splitting in some cases (e.g., ZnPh\(_4\)P\(^+\)) and a minor contribution in others (e.g., magnesium octaethylporphyrin cation radical, MgEt\(_8\)P\(^+\))\(^{27}\). Molecular orbital calculations suggest that radicals of this kind may occupy either of two nearly degenerate ground states, one of which provides for little spin density on meso carbon atoms and nitrogen atoms.\(^{27}\) Presumably, then, Chl\(^+\) belongs to this class of \(\pi\)-radical cations, and its epr line width depends mainly on hydrogen splittings with a small contribution from nitrogen. As an example of the effect of deuteration upon such a radical, the signal of MgEt\(_8\)P\(^+\) was narrowed by a factor of 2.4 after deuteration,\(^{27}\) and this conforms to the observations\(^{9,15,35}\) with deuterated Chl and bacteriochlorophyll.

Fig. 4 presents a comparison of the difference spectrum of Chl \(a\) and Chl\(^+\) with that of resting (dark) and photosynthesizing chloroplasts. The bleached form of P700, which is implicated in the photosynthetic mechanism and which is thought\(^{11,12,17}\) to be oxidized chlorophyll \(a\) in a special environment, possesses optical and epr spectral features closely congruent to those of the ethyl chlorophyllide \(a\) radical cation (ref. 26 and Fig. 2a) and of Chl\(^+\) (ref. 38). From this comparison and for the other reasons discussed, we conclude that oxidized P700 contains, indeed, a \(\pi\)-cation radical of the chlorin moiety of chlorophyll \(a\).

We are grateful to G. Sherman for kindly providing us with highly purified samples of chlorophyll \(a\) and of zinc tetraphenylchlorin.

Abbreviations: Chl, chlorophyll; ZnPh\(_4\)P\(^+\), zinc tetraphenylporphyrin cation radical; see, saturated calomel electrode; epr, electron paramagnetic resonance.

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There is of g-values reflects Nature, 206, 3451 (1965).

Recentlv Chibisov [Photochem. Photobiol., 10, 331 (1969)] published difference spectra of the photooxidation product of Chl a obtained by flash photolysis, and these (his Fig. 6) also resemble our data. The difference peak seen at 700 nm in chloroplasts is also shifted to 670 nm and may reflect the modified environment of Chl +* in the photoreaction center. Our observations further suggest that difference spectra of in vivo systems will also show substantial changes in the near infrared (about 820 nm).

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