Proceedings of the National Academy of Sciences
Vol. 67, No. 2, pp. 813–820, October 1970

The π-Cation Radical of Chlorophyll *a**

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Communicated by Donald D. Van Slyke, May 14, 1970

Abstract. Chlorophyll *a* 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 ± 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 π-cation radicals of porphyrins and chlorins, the chlorophyll radical is also identified as a π-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 π-cation radical of the chlorin component of chlorophyll *a*. 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[1]) might be due to removal of an electron from chlorophyll (Chl) or bacteriochlorophyll, or from closely related pigments.[2–6] 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 (P870) in the case of bacteria.[11–19]

It is established that oxidation of Chl by ferricyanide produces optical changes (bleaching of the ~700 nm absorption band) and epr signals (g = 2.0025 ± 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.[2,3,13,20]

Addition of ferric chloride to methanolic solutions of chlorophyll *a* 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 *a*, 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 ($Chl^{+\cdot}$) of chlorophyll *a*,

(*B*) subsequent attack on the $Chl^{+\cdot}$ by a nucleophile to form a neutral radical (the nucleophile might be chloride or methanol[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.[24] The feature distinguishing $Chl^{+\cdot}$ from the remaining possibilities is the positive charge, and preliminary moving boundary experiments indicated possible formation of $Chl^{+\cdot}$.[21] Later Stanienda demonstrated,[25] by cyclic voltammetry, two successive oxidations at a platinum electrode:

$$Chl \rightarrow Chl^{+\cdot} + e^- \qquad E_{1/2} = 0.52 \text{ V},$$

$$Chl^{+\cdot} \rightarrow Chl^{2+} + e^- \qquad E_{1/2} = 0.77 \text{ V [versus sce (aq)]}.$$

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[23] that the bleached species is formed by solvent attack upon $Chl^{+\cdot}$.

We recently reported[26,27] the existence of stable π-cation radicals and π-dications 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[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[21–23] or ferric perchlorate.[28]

In fact, the absorbance changes produced[26] by univalent oxidation of ethyl chlorophyllide *a* closely resemble the difference spectra reported for light-exposed chloroplast preparations.[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.[27] For charge-transfer reactions, a quartz epr tube was part of an ampoule with two side arms, one of which contained zinc tetraphenylporphyrin perchlorate ($ZnPh_4P^{+\cdot}ClO_4^-$) 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 $Chl^{+\cdot}$ (without carrier electrolyte) in order to minimize decomposition. $Chl^{+\cdot}$ was prepared by the charge-transfer reaction:

$$ZnPh_4P^{+\cdot} + Chl \rightarrow ZnPh_4P + Chl^{+\cdot} \qquad (1)$$

since the stable $ZnPh_4P^{+\cdot}$ cation radical has a higher potential [$E_{1/2}(1) = +0.71$ V] (ref. 27) than that required for oxidation of Chl *a* [$E_{1/2}(1) = +0.52$ 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_2Cl_2 (d = 1.31 g/cm³ at 25°C), $ZnPh_4P^{+\cdot}ClO_4^-$ was introduced into the arm which would become the anodic side, and both arms were filled with deaerated dimethylformamide (d = 0.94 g/cm³ 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 CH_2Cl_2 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 CH_2Cl_2, and an aliquot of a deaerated CH_2Cl_2 solution of Chl, partially titrated with $ZnPh_4P^{+\cdot}$ ClO_4^-, 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 CH_2Cl_2 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 ($E_{1/2}$ = 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. 2*a*). 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 CH_2Cl_2 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 $ZnPh_4P^{+\cdot}$ to Chl. After

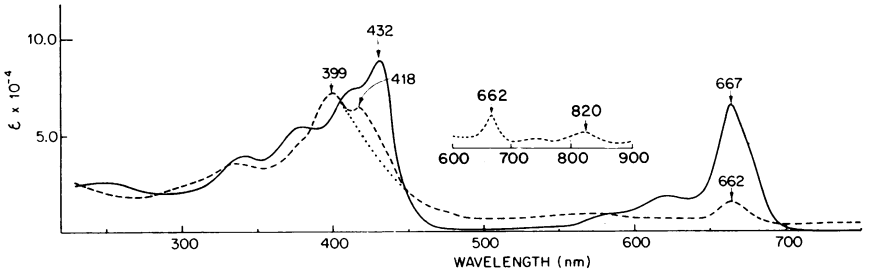

FIG. 1. Absorption spectra of chlorophyll *a* in CH_2Cl_2 (——), and its one-electron oxidation product (- - -), corrected for decomposition product (· · ·).

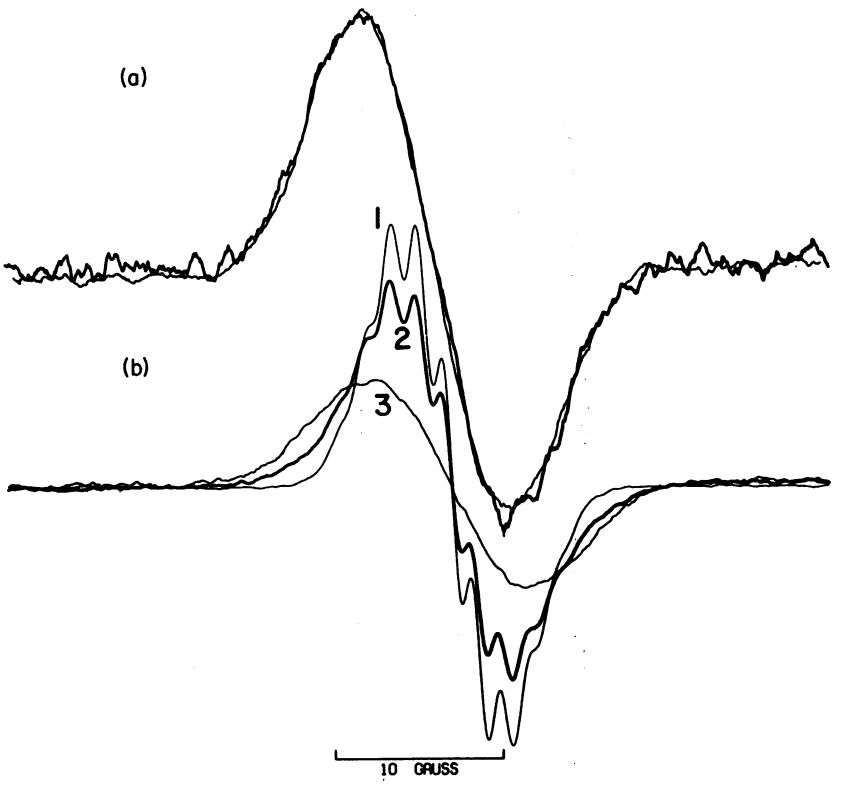

FIG. 2. (*a*) Epr spectra of the one-electron oxidation products of chlorophyll *a* and ethyl chlorophyllide *a* (*lighter tracing*) in butyronitrile. Electrolysis *in situ* at −50°C Mod. ampl. 1 G.

(*b*) Epr spectra of $ZnPh_4P^{+\cdot}$ ClO_4^- in CH_2Cl_2 titrated with chlorophyll *a* at 22°C. (1): $ZnPh_4P^{+\cdot}$; (2) $ZnPh_4P^{+\cdot}$ and $Chl^{+\cdot}$ present (about 3:2); (3) $Chl^{+\cdot}$. Mod. ampl. 0.5 G.

$ZnPh_4P^{+\cdot}$ ClO_4^- in CH_2Cl_2 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 $ZnPh_4P^{+\cdot}$ (ref. 27) was completely replaced by the characteristic singlet of $Chl^{+\cdot}$ (Fig. 2*b*).

With an excess of Chl, quantitation of free radicals by double integration of the first derivative spectra showed complete conversion of $ZnPh_4P^{+\cdot}$ to $Chl^{+\cdot}$. Stoichiometric (1:1) charge transfer was obtained in toluene as well as in dichloromethane. In the former, $Chl^{+\cdot}$ 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 $Chl^{+\cdot}$ in CH_2Cl_2 followed second-order decay kinetics for about 20 min. The characteristic infrared bands of the chlorophyll radical that are not obscured by $ZnPh_4P$ 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 $ZnPh_4P^{+\cdot}$. 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 $ZnPh_4P$ was $< 2\%$.

Formation of a free radical from a known chlorin: Zinc tetraphenylchlorin 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 $ZnPh_4P^{+\cdot}$, agree with $Chl^{+\cdot}$ 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 ($\pm$0.03) V for tetraphenylporphyrins to 0.35 ($\pm$0.1) V for etioporphyrins and 0.27 ($\pm$0.03) V for the chlorins Chl *a* and *b*, pheophytin *a* and *b*, and zinc tetraphenylchlorin.[33] If this difference is examined, rather than the individual potentials, the influence of the metal substituent is minimized. It is Δ which is constant if the sites of electron

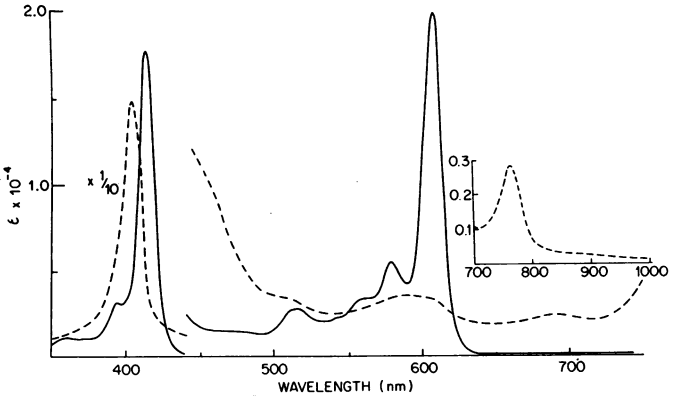

FIG. 3. Absorption spectrum of zinc tetraphenylchlorin (——) and its one-electron oxidation product (- - -).

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 π-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

$$(D)\ 2Chl^{+\cdot} = (Chl^{+\cdot})_2.$$

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_2Cl_2 may be due to a disproportionation reaction followed by nucleophillic attack on the *dication*

so formed, namely,
$$2Chl^{+\cdot} \rightleftarrows Chl + Chl^{2+} \quad (2)$$

followed by
$$Chl^{2+} + HOH \xrightarrow{\text{fast}} Chl(OH)^+, \quad (3)$$

and
$$Chl(OH)^+ \rightarrow \text{allomers}. \quad (4)$$

These reactions[34] 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 observed[32] with magnesium or zinc tetraphenylporphyrins, namely, $ZnPh_4P^{2+} + CH_3OH \rightarrow ZnPh_4P(OCH_3)^+ + H^+$. Also, under the conditions encountered in our experiments (and in methanol[28]), attempts to oxidize $Chl^{+\cdot}$ to the dication led only to allomerized material.

Beinert and Kok inferred[19] 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.[1–3,5,9,13,15,18,36,37] 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 π-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 chlorella[9] and purple photosynthetic bacteria[9,15] are narrowed by 2–3 times after deuterium substitution. This was interpreted[9,15] as due to magnetic interactions with hydrogens, and is consistent with delocalization into a π-system.[35]

Kohl *et al.* concluded[9] 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.[15] 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., $ZnPh_4P^{+\cdot}$) and a minor contribution in others (e.g., magnesium octaethylporphyrin cation radical, $MgEt_8P^{+\cdot}$)[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, $Chl^{+\cdot}$ belongs to this class of π-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 $MgEt_8P^{+\cdot}$ 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.

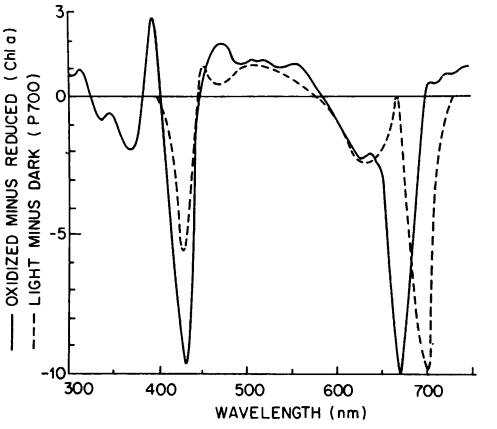

FIG. 4. Comparison of the optical difference spectrum of Chl and $Chl^{+\cdot}$ *a* with that reported from chloroplasts (data from ref. 30). Spectra have been normalized so that ~700 nm bands are equal.

Fig. 4 presents a comparison of the difference spectrum of Chl *a* and $Chl^{+\cdot}$ with that of resting (dark) and photosynthesizing chloroplasts. The bleached form of P700, which is implicated in the photosynthetic mechanism and which is thought[11,13,17] 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. 2*a*) and of $Chl^{+\cdot}$ (ref. 38). From this comparison and for the other reasons discussed, we conclude that oxidized P700 contains, indeed, a π-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; $ZnPh_4P^{+\cdot}$, zinc tetraphenylporphyrin cation radical; sce, saturated calomel electrode; epr, electron paramagnetic resonance.

* This research was supported in part by the U.S. Atomic Energy Commission, in part by the National Institutes of Health (grants AM14343 and AM14344), and in part by the Research Corporation.

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[34] Using the half-wave potentials for $Chl^{+\cdot}$ and Chl^{2+}, the equilibrium constant for Eq. (2) is estimated to be 6×10^{-5}. If reaction (3) did not occur, approximately 1% of a 10^{-4} M Chl^{+} solution would be present as Chl^{2+}.
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[38] Recently 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 $Chl^{+\cdot}$ 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).