ELECTRON PARAMAGNETIC RESONANCE
OF CHLOROPHYLL-WATER AGGREGATES*

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An extensive literature now exists on the visible,† infrared (IR),‡ nuclear magnetic resonance (NMR),.§ and electron paramagnetic resonance (EPR)¶ spectroscopy of chlorophyll solutions. Although much has been learned, the data are in many respects confusing and contradictory. The ambiguities in interpretation, which are particularly serious for the visible and EPR spectra, result primarily from the inability to specify the chlorophyll species responsible for the phenomena. For such information to be useful for the study of chlorophyll in nature, it is clearly of great importance to be able to correlate the various spectral properties of defined solutions with particular chlorophyll species.

Infrared and NMR investigations have established that the coordination number of the central magnesium atom of chlorophyll is larger than 4. The dihydrouporphyrin macrocycle itself is a tetradentate ligand, and the only way a coordination number of 5, or perhaps 6, can be attained by the magnesium atom is by a chlorophyll-nucleophile interaction, where the electron-donor molecule may be the solvent, another chlorophyll molecule, or a Lewis base (L). In electron-donor solvents such as alcohols, ketones, ethers, and the like, chlorophyll exists in monomeric form, with coordinated solvent in the axial position(s). In nonpolar solvents, in the absence of extraneous nucleophiles, the coordination unsaturation of the magnesium is relieved by coordination of the ketone oxygen function of one molecule of chlorophyll to the magnesium atom of another chlorophyll molecule. Nucleophiles added to such solutions may compete for coordination sites, and the chlorophyll species then present will be determined by equilibrium considerations involving the basicity of the nucleophile, the concentrations of chlorophyll and nucleophile, and the temperature. Because water not only can function as an electron donor, but also at the same time can form hydrogen bonds, and because of its small steric requirements, water is a unique nucleophile for chlorophyll. Equally important, the extent to which the hydrogen-bonding options of chlorophyll-bound water are exercised turns out to be very different for the various nonpolar solvents that can be used, and thus the effects of water may be very different in different media.

Hydration of Chlorophyll.—Chlorophyll a prepared by the usual methods¶ contains sizeable amounts of water, some as water of hydration and some occluded. Anhydrous chlorophyll a can be prepared on the vacuum line by repeatedly dissolving the chlorophyll in benzene or carbon tetrachloride and distilling the solvent away, followed by heating the chlorophyll film so obtained at 63°C for one hour in a high vacuum. (Even heating chlorophyll a at a pressure of 10⁻⁵ mm Hg for four hours at 80°C does not degrade it, as established by a chromatographic criterion.) No water can be detected by IR in chlorophyll so dried, in
agreement with the conclusions of Kutyurin and Knyazev.\(^5\) Hydrated chlorophyll \(a\) is obtained from dry chlorophyll by exposure of a film to water vapor (20 mm Hg). Chlorophyll \(a\) hydrates much more rapidly than does chlorophyll \(b\), pyrochlorophyll \(a\), or bacteriochlorophyll. Hydrated chlorophyll \(b\) loses water on exposure to ordinary air.

Anhydrous chlorophyll \(a\) dissolves freely in nonpolar solvents including aliphatic hydrocarbons such as \(n\)-hexane. Hydrated chlorophyll is soluble with rather more difficulty in hydrocarbon solvents. Ultrasonication is an effective procedure for preparing solutions of hydrated chlorophyll. A 10\(^{-3}\) \(M\) solution prepared by ultrasonication of hydrated chlorophyll \(a\) in water-saturated dodecane is clear to the eye, shows no background scattering in the visible region of the spectrum, and is stable indefinitely. Alternatively, dry chlorophyll can be dissolved in the dry hydrocarbon, and water introduced in a stream of nitrogen gas. To a good approximation, the properties of hydrated chlorophyll in aliphatic or alicyclic hydrocarbon solvents do not appear to be a sensitive function of the chlorophyll-water ratio, at least for the properties we have studied, and solutions of the same general composition as "wet" chlorophyll in aliphatic hydrocarbons have much the same properties regardless of the details of solution preparation. Addition of sufficient water to form a separate phase will eventually result in the partial or complete separation of the chlorophyll.

**Chlorophyll-Water Equilibria.**—Pertinent to the present discussion is an excellent study on chlorophyll-water interactions carried out by Rappaport and Van Winkle.\(^6\) This work was confined exclusively to \(CCl_4\) solutions, and consequently the solvent dependence of the chlorophyll-water interaction was not observed. Where there is overlap with the present work, the results are in excellent agreement, and the data of Rappaport and Van Winkle\(^6\) are entirely compatible with our equilibrium treatment. Sherman and Wang\(^2\) have recently studied chlorophyll-water interactions by IR and visible absorption spectroscopy, but they assumed that their studies were carried out with solid chlorophyll and therefore were unable to interpret their results in equilibrium terms.

In benzene, carbon tetrachloride, or chloroform, we find water to be a typical nucleophile, which interacts with chlorophyll dimers (at chlorophyll concentrations less than about 0.01 \(M\)) to form dimer and monomer solvates:

\[
\text{Chl}_2 + \text{H}_2\text{O} \rightleftharpoons \text{Chl}_2 \cdot \text{H}_2\text{O}, \quad (1)
\]

\[
\text{Chl}_2 \cdot \text{H}_2\text{O} + \text{H}_2\text{O} \rightleftharpoons 2\text{Chl} \cdot \text{H}_2\text{O}, \quad (2)
\]

\[
\text{Chl}_2 + 2\text{H}_2\text{O} \rightleftharpoons 2\text{Chl} \cdot \text{H}_2\text{O}. \quad (3)
\]

At low water and high chlorophyll concentrations, chlorophyll dimer monohydrate appears to be the principal species, but where the amount of water is comparable to or larger than the amount of chlorophyll, the equilibria are displaced in the direction of \(\text{Chl} \cdot \text{H}_2\text{O}\). In concentrated (>10\(^{-3}\) \(M\)) solutions of chlorophyll in these solvents, it is not possible to introduce sufficient water to disaggregate chlorophyll dimers to a significant extent, but in very dilute chlorophyll solutions (<10\(^{-4}\) \(M\)), a saturated solution of water in \(CCl_4\) (0.008 \(M\)) or of water
in benzene (0.03 M) is sufficient to convert a large part of the chlorophyll to Chl·H₂O.

To this point, the chlorophyll-water equilibria shown are identical with those postulated for any Lewis base. However, in aliphatic or alicyclic hydrocarbon solvents (dodecane, hexadecane, cyclohexane, or alkyl cyclohexanes), nucleophilic attack by water on the magnesium of chlorophyll has consequences different than those so far observed for any other nucleophile, and we find it necessary to postulate an additional equilibrium:

$$n(\text{Chl} \cdot \text{H}_2\text{O}) \rightleftharpoons (\text{Chl} \cdot \text{H}_2\text{O})_n.$$  \hspace{1cm} (4)

In hydrocarbon solvents, equilibrium (4) is displaced far to the right, and chlorophyll-water aggregates of colloidal dimensions predominate. Presumably, this equilibrium would also be important for alcohol ligands,\(^8\) which can also form hydrogen bonds, if steric factors did not interfere.

The magnitude of the equilibrium constant for reaction (4) is highly solvent-dependent. In benzene, carbon tetrachloride, or chloroform, the constant must be small. In solvents with poor solvation characteristics for the dihydrorophyrin macrocycle, the equilibrium constant for reaction (4) appears to become larger, and in long-chain aliphatic hydrocarbon solutions, equilibrium (4) is displaced far to the right. The driving force for the formation of chlorophyll-water aggregates in hydrocarbon solvents is in part that water coordinated to the central magnesium atom of one chlorophyll molecule can form hydrogen bonds with the Ring V ketone oxygen and carbomethoxy carbonyl oxygen functions of another molecule, and in part that the chlorophyll is oriented to maximize solvation of the phytol chains. Because both the chlorophyll and the water have two sites available for binding, repetition of the interaction leads to the large aggregates that are described in more detail below.

**Infrared Spectra.**—The infrared spectra of dry chlorophyll a in nonpolar solvents generally are characterized by an absorption peak at \(\sim 1653 \text{ cm}^{-1}\) in CCl₄ or benzene and at 1653–1600 cm\(^{-1}\) in aliphatic hydrocarbons, which has been assigned to C=O ketone oxygen coordinated to magnesium: C=O···Mg. As this is the interaction that leads to dimer formation, the peak at \(\sim 1650 \text{ cm}^{-1}\) has been termed an aggregation peak; the short-wavelength shoulder in aliphatic hydrocarbon solvents can be taken to indicate the presence of higher aggregates. Free ketone carbonyl absorption is found at 1695 cm\(^{-1}\), and ester carbonyl absorption at 1735 cm\(^{-1}\). Hydrated chlorophyll a in CCl₄ or benzene solution shows the typical dimer IR spectrum, but with increased absorption at 1695 cm\(^{-1}\) indicative of some disaggregation to monomer (equilibrium(3)). In aliphatic or alicyclic hydrocarbon solutions of hydrated chlorophyll, however, a strong absorption maximum is observed at 1638 cm\(^{-1}\), which is no doubt similar to the absorption peak of hydrated chlorophyll in Nujol observed by Sherman and Wang,\(^7\) but which was not differentiated by them from the 1650–1660 cm\(^{-1}\) aggregation peak. In our solutions of hydrated chlorophyll in aliphatic hydrocarbon solvents, the 1695 cm\(^{-1}\) absorption peak is very weak, which we take to indicate only a small amount of free carbonyl in the absorbing species. We therefore assign the absorption peak at 1638 cm\(^{-1}\) to Ring V ketone oxygen
which is hydrogen-bonded to water coordinated to the central magnesium atom of another chlorophyll molecule: \( \text{C} = \text{O} \cdots \text{H} \cdots \text{O} \cdots \text{Mg} \). This assignment follows from the near absence of free keto carbonyl in the IR spectrum, from evidence in the NMR spectrum that the water is bound to the chlorophyll, and from the water spectrum in the OH stretch region. Three absorption peaks corresponding to water coordinated to magnesium, to hydrogen-bonded ester carbonyl, and to hydrogen-bonded keto carbonyl can be seen in the OH stretch region of the IR (3600 cm\(^{-1}\), 3460 cm\(^{-1}\), 3250 cm\(^{-1}\)), and these correspond very well to the assignments made in the 1600–1800 cm\(^{-1}\) region of the IR spectrum.

Studies with models show that a water molecule coordinated to the magnesium atom of one chlorophyll molecule can be oriented to form hydrogen bonds simultaneously to the ketone oxygen (at position C-9) and carbomethoxy (at C-10) carbonyl functions of another chlorophyll molecule. Participation of this ester carbonyl is established by the splitting of the ester carbonyl absorptions into two peaks now appearing at 1743 and 1727 cm\(^{-1}\). The interaction described here is compatible with the absolute configuration of chlorophyll established by Fleming.\(^9\) Thus, the 1660 cm\(^{-1}\) peak (chlorophyll n-mer), the 1653 cm\(^{-1}\) peak (chlorophyll dimer), and the 1638 cm\(^{-1}\) peak (chlorophyll-water aggregates) are aggregation peaks, but their origin and significance are quite different.

**State of Ligand Water.**—We have found evidence from NMR that water coordinated to magnesium is more acidic than bulk water. The coordination to magnesium deshields water bound by chlorophyll in CHCl\(_3\) solution in a fashion very similar to that described by Nakamura and Meiboom,\(^10\) who found the pK\(_a\) of methanol to increase 1000-fold by coordination to Mg\(^{++}\). The hydrogen of chlorophyll-bound water must be relatively acidic, and because it has been noted that increase in acidity parallels hydrogen-bond stability,\(^11\) it is reasonable to assume that the strong hydrogen bonds formed by water coordinated to magnesium are related to the increased acidity of the bound water.

**Chlorophyll-Water Aggregates.**—Chlorophyll-water aggregates of colloidal dimensions exist in solutions of hydrated chlorophyll in water-saturated aliphatic hydrocarbons, as is readily demonstrated by ultracentrifugation and X-ray diffraction experiments. Neither dry nor hydrated chlorophyll dissolved in CCl\(_4\) or benzene (5 \( \times \) 10\(^{-4}\) \( \text{M} \)) can be sedimented by even very long-continued centrifugation. However, hydrated chlorophyll dissolved by ultrasonication in butyl cyclohexane or hexadecane is completely sedimented by ultracentrifugation at 130,000 \( \times \) g for 30 minutes. X-ray diffraction examination of hydrated chlorophyll solutions in hydrocarbon solvents reveals the presence of entities with considerable order. Whereas solutions of dry chlorophyll (10\(^{-2}\) \( \text{M} \)) in butyl cyclohexane, dodecyl cyclohexane, or hexadecane show no X-ray reflections, solutions of hydrated chlorophyll (10\(^{-2}\) \( \text{M} \)) in the same solvents give a sharp X-ray diffraction line indicative of a spacing of 7.5 Å. This distance is very close to that estimated from models for the Mg–Mg distance in two parallel chlorophyll molecules separated by a water molecule.

Figure 1 shows two possible representations of chlorophyll-water aggregates.
Based on the X-ray and IR data, the phytanyl side chains are shown one above the other in each configuration, but the models also permit a spiral configuration for the phytanyl chains. In Figure 1b, a micelle is shown with dihydroporphyrin rings oriented to form an inner hydrophilic core. Although the micelles must have a considerable degree of order, it is likely, as in the case of micelles in aqueous systems, that they exhibit a distribution of sizes and compositions.

Absorption Spectra in the Visible.—Jacobs, Vatter, and Holt, and Sherman and Linschitz, among many others, observed a general relationship between the absorption spectra in the visible and chlorophyll hydration. The dependence of the electronic transition spectra of chlorophyll in water can now be recognized explicitly as a correlation between \( \lambda_{\text{max}} \) (red) and the presence in solution of \((\text{Chl-H}_2\text{O})_n\) aggregates. Data on the visible absorption spectra of dry and hydrated chlorophylls as a function of solvent are shown in Table 1. Solutions of chlorophyll \( b \) and bacteriochlorophyll in aliphatic hydrocarbon solvents show similarly large red-shifts on hydration.

**Table 1. Spectral properties of dry and hydrated chlorophylls in benzene and cyclohexane solutions.**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solvent</th>
<th>( \lambda_{\text{max}} ) (blue)</th>
<th>( \lambda_{\text{max}} ) (red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Chl a</td>
<td>( \text{C}_6\text{H}_6 )</td>
<td>433</td>
<td>666, 673*</td>
</tr>
<tr>
<td></td>
<td>( \text{C}<em>6\text{H}</em>{12} )</td>
<td>429</td>
<td>662, 673*</td>
</tr>
<tr>
<td>Hydrated Chl a</td>
<td>( \text{C}_6\text{H}_6 )</td>
<td>432</td>
<td>666</td>
</tr>
<tr>
<td></td>
<td>( \text{C}<em>6\text{H}</em>{12} )</td>
<td>430, 446</td>
<td>665(w), 743</td>
</tr>
<tr>
<td>Dry Chl b</td>
<td>( \text{C}_6\text{H}_6 )</td>
<td>453</td>
<td>643, 662</td>
</tr>
<tr>
<td></td>
<td>( \text{C}<em>6\text{H}</em>{12} )</td>
<td>453</td>
<td>643, 662</td>
</tr>
<tr>
<td>Hydrated Chl b</td>
<td>( \text{C}_6\text{H}_6 )</td>
<td>453</td>
<td>643</td>
</tr>
<tr>
<td></td>
<td>( \text{C}<em>6\text{H}</em>{12} )</td>
<td>453</td>
<td>643(w), 684</td>
</tr>
<tr>
<td>Dry BChl†</td>
<td>( \text{C}_6\text{H}_6 )</td>
<td>360, 395</td>
<td>778</td>
</tr>
<tr>
<td></td>
<td>( \text{C}<em>6\text{H}</em>{12} )</td>
<td>360, 395</td>
<td>775</td>
</tr>
<tr>
<td>Hydrated BChl†</td>
<td>( \text{C}_6\text{H}_6 )</td>
<td>360, 395</td>
<td>778</td>
</tr>
<tr>
<td></td>
<td>( \text{C}<em>6\text{H}</em>{12} )</td>
<td>360, 395</td>
<td>775, 865</td>
</tr>
</tbody>
</table>

* Shoulder.
† Bacteriochlorophyll.
Electron Paramagnetic Resonance Spectra.—Because there is a widespread conviction that the long-wavelength absorbing forms of chlorophyll are important in photosynthesis,\textsuperscript{10} it becomes of interest to see whether the long-wavelength absorbing chlorophyll-water aggregates are photoactive. We have now examined the EPR of dry and hydrated chlorophyll \textit{a} in various nonpolar solvents and can establish a clear-cut relationship between their photoinduced EPR signals in the presence of chlorophyll-water aggregates. We are also able to show that the dark signal so often observed in chlorophyll preparations is very likely a result of exposure to oxygen. Very dry chlorophyll \textit{a} solutions in nonpolar solvents show neither dark signals nor photoinduced signals. Dry films of chlorophyll likewise show neither dark nor photoinduced signals. Hydrated chlorophyll in an aliphatic hydrocarbon solution prepared with rigorous exclusion of oxygen shows no dark signal and produces an EPR signal on irradiation with red light, which completely decays on standing in the dark.

All EPR spectra were measured on a Varian V-4502 spectrometer with a 12-in. magnet and 100-kHz modulation. Modulation amplitude varied from 0.5 to 1.0 gauss. The \textit{g} values were determined from the magnitude of the magnetic field, which was determined by a calibrated Varian F-22 gaussmeter, and the microwave frequency at resonance, measured directly with a 5445 L Hewlett-Packard counter and frequency converter. All EPR samples were made up in quartz sample tubes terminated with vacuum-tight greaseless O-ring joints for attachment to a high vacuum system. After preparation, samples were sealed off under continuous pumping. Chlorophyll was thoroughly dried by codistillation with CCl\textsubscript{4} and heating at 60\deg C in vacuum. Reagent grade solvents were mixed with concentrated sulfuric acid for several hours, washed with sodium carbonate solution and water, and then dried and stored over silica gel. Oxygen-free solutions could be prepared on the vacuum line with dodecane, which is sufficiently volatile to be transferred by high vacuum distillation and condensed onto oxygen-free dry or hydrated chlorophyll.

Figure 2 shows EPR spectra of dry and hydrated chlorophyll \textit{a} and deuterio-
Table 2. Photoinduced* EPR signals from chlorophyll-water aggregates.

<table>
<thead>
<tr>
<th>System†</th>
<th>Line width (gauss)</th>
<th>g Value (± 0.0002)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H Chl a-H2O</td>
<td>1.0</td>
<td>2.0025</td>
</tr>
<tr>
<td>D Chl a-H2O</td>
<td>0.6</td>
<td>2.0028</td>
</tr>
<tr>
<td>H Chl a-D2O</td>
<td>1.3</td>
<td>2.0026</td>
</tr>
<tr>
<td>D Chl a-D2O</td>
<td>0.6-0.7</td>
<td>2.0026</td>
</tr>
<tr>
<td>H Chl a-H2O17</td>
<td>3.0</td>
<td>2.0028</td>
</tr>
</tbody>
</table>

* Irradiated with >600 nm red light obtained from a Xenon lamp and a Corning sharp-cutoff filter no. 2434. There is essentially zero radiation with wavelength shorter than 570 nm. Infrared radiation is eliminated by a filter in the light source.

† 0.001 M solutions in dodecane.

chlorophyll a in dodecane solution. The dry solutions show no EPR signal with red light (>600 nm), but the hydrated solutions show a photosignal that begins to appear as soon as the light is turned on and that disappears on standing in the dark. Isotopic substitution was used to study the nature of the photoinduced signal (Table 2). Deuterio-chlorophyll16 hydrated with either H2O or D2O gives a distinctly narrower signal than does ordinary hydrogen chlorophyll.

Figure 3 shows the effect of temperature on the EPR signal photoinduced by red light in a hydrated chlorophyll a solution in dodecane. When the solution is prepared with careful exclusion of oxygen, no dark signal is observed. On illumination at 20°C, the characteristic photosignal appears. The sample, still in the light, is heated to 60°C (where, from IR and visible absorption spectroscopy, it is known that the chlorophyll-water aggregates are dissociated), and the EPR signal disappears. On cooling and reconstituting the aggregate, a photosignal begins to reappear around 40°C and fully reappears at 20°C.

If the hydrated chlorophyll solutions are prepared with oxygen present, then the situation is quite different, and the results are about the same as those already often reported for solid17,18 or chloroplastic preparations.19 Hydrated chlorophyll a in dodecane solutions exposed to oxygen shows a persistent narrow, dark signal that is almost certainly associated with a nonphoto-lytic chemical reaction. These dark signals can be very intense, depending upon details of sample preparation, but are little affected by subsequent irradiation. Thus hydrated chlorophyll in oxygen-containing dodecane or hexadecane gives rise to at least two kinds of EPR signals, a dark signal and a superimposed photosignal, but dry chlorophyll a in aliphatic or alicyclic hydrocarbon solutions free of oxygen gives neither a light nor a dark signal.

All of the observed EPR signals appear to arise from free radicals with g values close to that of the free electron. There are clearly a number of oxidized
or reduced chlorophyll species that could produce such a signal. The origin of the signal is consistent with the idea of a H\textsuperscript{+} transfer (or abstraction) from coordinated water hydrogen-bonded to the C-9 keto group. In addition, the relatively narrow width of the signal suggests possible proton exchange between the keto oxygen and the coordinated water oxygen, with the formation of radical anions and cations of chlorophyll. The narrowing of the signal observed with deuterio-chlorophyll indicates that the electron has been delocalized into the π-electron system of the macrocycle. The signal broadening resulting from H\textsubscript{2}O\textsuperscript{17} suggests that the observed signal is composite and contains a contribution from an electron localized near the water oxygen atom.

* Based on work performed under the auspices of the United States Atomic Energy Commission.
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