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CHLOROPHYLL–WATER INTERACTIONS
HYDRATION, DEHYDRATION AND HYDRATES OF CHLOROPHYLL

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SUMMARY

The interaction of water with chlorophyll may have far-reaching effects on the spectral properties of chlorophyll. We have devised a vapor phase chromatographic procedure for the determination of water in chlorophyll. The dehydration, hydration, and hydrates of chlorophylls *a* and *b* have been explored with the aid of this analytical procedure. Absorption spectra in the visible and infrared are presented for chlorophyll–water systems of known composition.

INTRODUCTION

That the coordination properties of magnesium are implicated in chlorophyll behavior appears to have been first suggested by EVSTIGNEEV *et al.*[1]. However, it is recent nuclear magnetic resonance (NMR)[2] and infrared[3–7] spectroscopic investigations that have provided a firm experimental basis for the view that the coordination properties of magnesium are a decisive factor in many chlorophyll phenomena[8]. As shown in the usual chlorophyll structural formula, magnesium has the coordination number 4. The evidence is quite conclusive, however, that at least one of the axial magnesium positions must always be occupied by an electron donor molecule. In nonpolar solvents, in the absence of extraneous nucleophiles, another chlorophyll molecule acts as the electron donor. The keto oxygen function of one chlorophyll molecule enters the coordination sphere of the magnesium atom in another chlorophyll molecule, and the Mg---O=C coordination interaction then results in the formation in these solvents of chlorophyll dimers[2,8] and oligomers[9]. Nucleophiles such as alcohols, ethers, ketones and the like can compete for the coordination site at magnesium to disaggregate the chlorophyll–chlorophyll aggregates. Thus, in polar solvents, chlorophyll as shown by NMR and infrared studies, occurs as the monomer mono- or di-solvate[9,10]. Water turns out to be a particularly interesting nucleophile for chlorophyll. By virtue of its molecular size, and its amphoteric nature as manifest by its ability to act both as an electron donor as well as to form concurrently two

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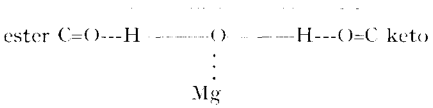
hydrogen bonds, water interacts with chlorophyll (in suitable nonpolar solvents) to form chlorophyll–water adducts of an unusual nature. These chlorophyll water adducts are held together by a water molecule coordinated to the central magnesium atom of one chlorophyll molecule and simultaneously hydrogen bonded to the keto and carbomethoxy carbonyl oxygen functions of another chlorophyll molecule:



$$\text{ester C=O---H}\text{------}\underset{\vdots \atop \text{Mg}}{\text{O}}\text{------H---O=C keto}$$

Repetition of this process results in aggregates or micelles of colloidal dimensions[11]. Chlorophyll–water micelles constituted by this repeating unit are observed to have an absorption spectrum strongly shifted to the red, and a narrow, completely reversible photo electron spin resonance (ESR) signal is obtained when the micelles are illuminated with red light[12]. The discovery of these chlorophyll–water micelles and their remarkable photo-activity have provided the basis for a new hypothesis for the primary light conversion step in photosynthesis[13].

There are clear intimations in the prior literature of the importance of chlorophyll–water interactions. Thus, Livingston and Weil[14] noted that chlorophyll dissolved in pure dry hydrocarbons "is (practically) non-fluorescent". The addition of water (or other bases) activated the fluorescence.

Jacobs *et al.*[15] found that water was required to produce "crystalline" chlorophyll; an X-ray diffraction pattern could only be obtained when the chlorophyll was precipitated in the presence of water. Other effects of water were also noted. Belovtseva *et al.*[16] and Anderson and Calvin[17] found that removal and addition of water affected the X-ray diffraction pattern of chlorophyll. Kholomogorov and Terenin[18] and Anderson and Calvin[17] noted that water and/or oxygen were involved in the production of a photo-ESR signal from solid chlorophyll. The infrared spectra of chlorophyll were likewise earlier noted to be sensitive to water by Karyakin *et al.*[19,20] and by Sidorov and Terenin[21]. More recently, Sherman and Wang[22] have again examined the effects of water on the infrared, visible absorption[23] and ESR[24,25] spectra of chlorophyll *a*. Sherman and Wang[22] confirmed and extended many of the earlier observations. Because no interpretation of the experimental results on the effects of water could be made, the common basis for the effects of water on the X-ray diffraction, visible, infrared, NMR and ESR spectra of chlorophyll noted by many of these investigators (and others not mentioned here) was not fully appreciated.

The basic facts about the hydration, dehydration, and hydrates of the chlorophyll were also not established, and there are conflicting statements in the literature about the nature and composition of chlorophyll hydrates[26,28].

The present investigation shows that chlorophyll prepared by the recipes of Jacobs *et al.*[15] and Anderson and Calvin[29], so-called "crystalline" chlorophyll, approximates the composition of the monohydrate, $\text{Chl}\cdot\text{H}_2\text{O}$. X-ray diffraction data in the literature[15,16,30] thus are actually those for $(\text{Chl}\cdot\text{H}_2\text{O})_n$ micelles. The effects of water are especially important in the visible absorption spectroscopy of chlorophyll, and such experiments require careful control of the water content of the system to be interpretable.

EXPERIMENTAL

Chlorophyll

The chlorophyll used in these experiments was prepared by the method of STRAIN AND SVEC[31]. In this procedure, the chlorophyll is precipitated in the presence of water and the product generally pumped in a 10^{-2} mm vacuum at room temperature.

Vapor phase chromatographic procedure for water in chlorophyll

The method depends on the separation of water on a Porapak Q column (Brochure, Waters Associates, Framingham, Mass., U.S.A.) and its detection by a thermal conductivity cell. The use of the organic polymer together with a shorter than usual column eliminates the usual tailing of the water peak commonly encountered when using vapor phase chromatography for the quantitative estimation of water. The smallest amount of water we can measure with confidence is about 0.2 μg, or 1 ppm of H_2O given a sample volume of 200 μl. Because nonpolar solvents appear considerably later on a Porapak Q column than the water, there is no interference in the water determination by a large solvent peak. To determine the water content of a chlorophyll sample, the chlorophyll is dissolved in a suitable solvent, generally benzene or cyclohexane containing a known amount of an internal standard, and the chlorophyll solution or aliquots of it are injected into the gas chromatograph.

A Varian Autoprep Model A 700 gas chromatograph with a W-X rhenium–tungsten thermal conductivity detector equipped with a recorder with full-scale deflection of 1 mV was used. The thermal conductivity filaments were operated at 250 mA. A 40-cm by 6-mm inner diameter stainless steel column was packed with uncoated Porapak Q. The oven temperature was maintained at 110°; the injection port and the detector temperatures were 180° and 130° respectively. Helium gas was used as the carrier at a head pressure of 3.2 atm and a flow rate of approx. 40 ml/min; the optimum gas flow is determined for each new column. A 60-cm drying tube containing Linde Molecular Sieve 5A was positioned between the helium cylinder and the gas inlet of the chromatograph. To stabilize operation conditions, the instrument was not shut down overnight or weekends.

A careful investigation was made to ascertain the optimum injection port temperature. If the temperature is too low, ligands coordinated to the chlorophyll may not be liberated. If the temperature is too high, the chlorophyll may be pyrolyzed and a methanol peak may be detected from the decarbomethoxylation[32] or other decompositions of the chlorophyll. Thermal decomposition of chlorophylls *a* and *b* with evolution of volatile products becomes significant at injection port temperatures above 200°. The injection port temperature was set by determining the recovery of known amounts of water and acetone added to anhydrous solutions of chlorophyll in benzene. In the range 180–200°, recovery is complete and interference by decomposition products of the chlorophyll can be neglected. The quantitative recovery of water indicates that chlorophyll does not bind water significantly under our operating conditions.

To render the procedure quantitative, standard solutions are used for calibration. Benzene was dried with silica gel and calcium hydride. Acetone was dried over silica gel and was added as internal standard to dry benzene to make a solution 0.04% by weight. Acetone is preferred to ethanol as internal standard, as we have observed alcohololysis of chlorophyll in the presence of ethanol. Known amounts of water (0.005–0.04% by weight) were added, 50-μl samples were injected with a Hamilton constant rate syringe, and the relative peak heights of the acetone and water measured. Peak heights were used rather than peak areas because of the large errors inherent in measuring the half-widths of the very narrow water peaks. As a further check on the calibration procedure, the standard solutions were compared by vapor phase chromatography and Karl Fischer titration performed with a Beckman KF3 Aquameter. Agreement between the two procedures was excellent.

The chlorophyll sample is dissolved in 200 μl of dry benzene containing a known amount of acetone. In a duplicate determination two 50-μl portions of this solution were injected directly into the gas chromatograph. The volumes 200 and 50 μl were chosen to keep the relative errors caused by volume measurements and solution evaporation small. The standard deviation of our water determination, as determined with standard solutions containing 5.0 μg H_2O/50 μl is ± 0.15 μg H_2O; with a standard solution containing 0.9 μg H_2O/50 μl it is ± 0.17 μg H_2O. The pick-up or loss of water is the main source of inaccuracy when amounts as small as 1–10 μg of water are to be determined. We followed the standard precautions common in the ultramicroanalysis of water[33,34]. A nitrogen-purged glove-box was used for manipulations and transfers. Drying operations were carried out on a vacuum line. Solutions were kept in flasks with capillary necks fitted with Teflon stoppers. Though a water blank cannot be avoided, it must be kept constant and as low as possible. In our case the blank for all steps of the water determination was found to be from 1.0–1.8 μg H_2O/50 μl, depending mainly on the initial water content of the acetone-containing benzene used to dissolve the chlorophyll. For a given initial water content, the blank could be reproduced within ± 0.2–0.3 μg H_2O, thus allowing a water determination in a chlorophyll sample to within ± 8–10% for an amount of about 5 μg H_2O/50 μl. This amount of water is equivalent to a sample of about 1 mg chlorophyll $a \cdot$monohydrate dissolved in a 200 μl volume. For higher accuracy in measuring the chlorophyll–water mole ratio, about 3 mg Chl $a \cdot H_2O$ were dissolved in 150 μl of solvent, resulting in a sample containing about 20 μg H_2O/50 μl. In this case, the error of the blank contributes less to the overall relative error, which then drops to ± 5–6%.

It can be seen from the foregoing that a water determination on a chlorophyll sample, or in a solution even as dilute as 10^{-3} M, can be carried out with reasonable accuracy when the chlorophyll and water are present in equivalent amounts, and a sufficiently large sample is injected into the chromatograph. To prove that chlorophyll is anhydrous by the vapor phase chromatographic analytical technique is a rather different problem. A larger chlorophyll sample (5 mg) makes it possible to detect an amount of water equivalent to less than 1 mole % of the chlorophyll. Infrared spectroscopy in the water stretch region is a valuable adjunct to set a lower limit for the water content of a chlorophyll solution. By the use of concentrated chlorophyll solutions and a long light path (5 mg/ml, 10 mm quartz cell), in agreement with the observations of RAPPAPORT[35], we can show the solution to contain less than 10^{-5} M water. For a 10^{-2} M chlorophyll solution, this establishes a chlorophyll–water ratio of 1000:1.

RESULTS

Water content of chlorophyll as received

Chlorophyll prepared by the methods of STRAIN AND SVEC[31] is precipitated in the presence of water from aliphatic hydrocarbon solution. It is, therefore, not surprising that it contains water. Routine analysis of a series of chlorophyll preparations show the chlorophyll–water mole ratio to vary from 1:0.9 to 1:1.15 (Table I). The usual product is thus close in composition to $(\mathrm{Chl \cdot H_2O})_n$, and except in critical applications, can be taken as such. Equilibration of chlorophyll *a* in ordinary air gives the monohydrate very closely, and dehydrated chlorophyll exposed to the atmosphere will gradually reform the monohydrate.

TABLE I

ANALYSES OF CHLOROPHYLL *a*–WATER ADDUCTS

Samples were pumped at 10^{-2} Torr for the indicated time and temperature. Prep. 1 as received. Preps. 2 and 3: dried by co-distillation at room temperature with CCl_4, 3 times. Prep. 4: film deposited from carbon tetrachloride solution; hydrated with water vapor at 50° for 5 days. Preps. 5 and 6: solution in carbon tetrachloride hydrated with water vapor at 23° for 3 days. Prep. 7: solution in benzene hydrated with water vapor at 23° for 3 days. Preps. 8 and 9: hydrated in H_2O–cyclohexane emulsion at 23°, followed by evaporation with wet N_2. Prep. 10: hydrated by bubbling wet N_2 gas through *n*-octane solution for 20 h.

Prep. No.	*Sample treatment*		*Sample size in 50 μl benzene (μg chlorophyll a)*	*Water found (μg ± 0.5 μg)*	*Chlorophyll/H₂O (mole ratio)*
	Temp.	*Time (h)*			
1	23°	0.3	500	10.2	1:1.00*
2	23°	0.3	400	1.6	1:0.20
3	70°	2	5930	0.4	1:0.003
4	23°	24	275	6.4	1:1.15
5	23°	1	470	10.4	1:1.10
6	23°	24	220	4.7	1:1.05
7	23°	24	390	9.0	1:1.15
8	23°	1	275	4.6	1:0.85
9	23°	24	275	2.9	1:0.50
10	23°	4	590	13.5	1:1.15

* This sample had $A_{740\,m\mu}/A_{665\,m\mu} > 10$ in hexane solution.

Chlorophyll *b* is more variable in composition; in general as received it will have a water content lying between 3:2 and 2:1 (Table II). A chlorophyll *b* monohydrate forms with far more difficulty than is the case for *a*. Thus, in the case of chlorophyll *b*, it is preferable to carry out the hydration of the dried *b* under controlled conditions if a hydrate of known composition is desired.

Dehydration

Chlorophyll *a* can be thoroughly dried by the following procedure: the chlorophyll as received is dissolved in dry CCl_4, and the solvent evaporated at room temperature in a stream of dry nitrogen. This procedure is repeated 3 times. The chlorophyll is then heated at 60–80° in a good vacuum for 2 h. Column chromatography on powdered sugar of the product shows that no degradation of the chlorophyll

TABLE II

ANALYSES OF CHLOROPHYLL *b*-WATER ADDUCTS

Samples were pumped at 10^{-2} Torr for the indicated time and temperature. Preps. 1 and 2 as received. Preps. 3–6: dried by co-distillation at room temperature with CCl_4, 3 times. Preps. 7 and 8: film deposited from carbon tetrachloride solution hydrated with water vapor at 50° for 5 days. Preps. 9 and 10: solution in carbon tetrachloride hydrated with water vapor at 23° for 3 days. Preps. 11 and 12: solution in benzene hydrated with water vapor at 23° for 3 days.

Prep. No.	*Sample treatment*		*Sample size in 50 µl benzene (µg chlorophyll b)*	*Water found (µg ± 0.5 µg)*	*Chlorophyll b/H_2O (mole ratio)*
	Temp.	*Time (h)*			
1	23°	0.3	490	4.2	1:0.45
2	23°	0.3	380	4.5	1:0.60
3	23°	2	415	5.6	1:0.70
4	70°	2	415	2.9	1:0.35
5	50°	1	2480	1.8	1:0.04
6	70°	23	5120	1.0	1:0.01
7	23°	1	365	7.5	1:1.05
8	23°	24	555	6.5	1:0.60
9	23°	1.5	555	11.5	1:1.05
10	23°	24	385	6.9	1:0.90
11	23°	1	430	6.4	1:0.75
12	23°	24	430	6.1	1:0.70

occurs from this treatment. Analysis by vapor phase chromatography, confirmed by infrared studies, shows the chlorophyll *a* obtained by this drying procedure has a chlorophyll–water mole ratio of at least 100:1 (Table I). Anhydrous chlorophyll *a* is surprisingly soluble in aliphatic hydrocarbons, and an 0.1 M solution in *n*-hexane or dodecane can easily be formed. A film of such thoroughly dehydrated chlorophyll *a* is quite hygroscopic, and must be preserved from contact with air to prevent some water pick-up.

Chlorophyll *b* retains water much more tenaciously than does chlorophyll *a*; even after heating at 70° for 2 h in a good vacuum, a chlorophyll–water ratio of 1:0.31 is still observed (Table II). Co-distillation with CCl_4 or benzene at 60° under atmospheric pressure has been found to be a simple and reasonably effective way of removing water from chlorophyll *b*. The *b* is dissolved in a small amount of dry benzene, immersed in a 60° silicone oil bath and the solvent removed in a stream of dry nitrogen. This procedure is repeated 3 times. The film is then heated at 50° for 1 h at 10^{-2} mm. In our experience, the chlorophyll *b* obtained by this procedure has a chlorophyll *b*–water mole ratio of at least 25:1. For demanding applications, the dehydration procedure can be repeated several more times, and the chlorophyll *b* finally heated to 60–70° for several hours in a high vacuum. Anhydrous chlorophyll *b* is distinctly less soluble in aliphatic hydrocarbons than is *a*; $5 \cdot 10^{-2}$ M solutions of chlorophyll *b* in cyclohexane or *n*-butyl cyclohexane can be readily prepared, but the solubility of *b* in straight chain hydrocarbons is distinctly less.

Hydration

Chlorophyll can be hydrated either as a film or in solution. Films are prepared in glass vials from dry carbon tetrachloride solution by evaporation in a stream of dry nitrogen while rotating the vial. To achieve equilibration in a reasonable time, thin films are essential. We have found that a minimum water vapor pressure is required to effect hydration. Below a vapor pressure of 12 mm, chlorophyll films pick up no significant amounts of water. Hydration of chlorophyll *a* films proceeds fairly rapidly at 40–50°. Superficial water can then be removed by pumping in a vacuum for an hour at room temperature. These procedures produce a product with a chlorophyll–water ratio of $1:1 \pm 0.1$. Long continued pumping at room temperature does not remove further water; once the ($\mathrm{Chl \cdot H_2O}$) film is formed, it is stable to pumping even for 24 h.

A convenient procedure for the hydration of chlorophyll *a* in solution was devised. This procedure should be considered as a possible alternative to the "shaking with water" step in the conventional chlorophyll *a* preparation.

Chlorophyll is dried by CCl_4 co-distillation two times, and then dissolved in *n*-octane to give a 2–4 mM solution. Water-saturated N_2 gas is slowly bubbled through this solution for a minimum period of 8 h for every 10–20 mg chlorophyll *a* present. The chlorophyll *a* will precipitate as hydration proceeds. The hydration of chlorophyll *a* can be easily followed by observing the spectrum in the visible region*. As hydration occurs the 665/675-nm band of the anhydrous chlorophyll *a* decreases and the 743-nm band of the $(\mathrm{Chl}\ a \cdot H_2O)_n$ in the supernatant solution increases. When a ratio of about 10 for $A_{743\,\mathrm{m}\mu}/A_{665\,\mathrm{m}\mu}$ is reached, the hydration can be taken as completed. The solution is then centrifuged (10000 rev./30 min) and the precipitate washed at least 3 times with low boiling light petroleum (b.p., 30–60°) to remove the octane and any incompletely hydrated chlorophyll *a*. The final precipitate obtained by centrifugation under the conditions cited is then pumped on at room temperature in a 10^{-2} mm vacuum for at least 4 h. The chlorophyll *a* hydrate so obtained has a chlorophyll a/H_2O mole ratio of $1:1.0 \pm 0.1$.

Chlorophyll *a* can also be hydrated by slow evaporation from water-saturated CCl_4 or benzene. The solvents are allowed to evaporate slowly (2 or 3 days) under vacuum in a water-vapor saturated atmosphere. The films of hydrated chlorophylls are then pumped on at at least 10^{-2} Torr for 1 h. The hydrate so obtained has a chlorophyll–water mole ratio of $1:1 \pm 0.1$. This hydrate is likewise stable to prolonged pumping in vacuum at room temperature.

If the chlorophyll is evaporated from a carbon tetrachloride or benzene–water emulsion (in which there is a separate liquid water phase) then hydrates of variable composition are formed. It would appear that the state of chlorophyll in such solutions is not identical with that in water-saturated solvents where no excess liquid water is present. The hydrated chlorophyll obtained under these conditions does lose water on long-continued pumping, and products with a composition near 2:1 can be ob-

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* The absorption spectrum in the visible of an *n*-hexane solution can be taken as an easy check to determine whether a given chlorophyll preparation consists of the $(\mathrm{Chl}\ a \cdot H_2O)_n$, the anhydrous chlorophyll *a* or a mixture of both. Preparation of chlorophyll *a* $>10^{-4}$ M solution (cell <1 mm) and use of ultrasonication is advisable. If a too dilute solution is used, the following equilibrium will lead to erroneous conclusions about the state of the chlorophyll:

$$(\mathrm{Chl}\ a \cdot H_2O)_n \rightleftarrows n(\mathrm{Chl}\ a \cdot H_2O)$$

The 743-nm band will decrease and the band for monomeric $\mathrm{Chl}\ a \cdot H_2O$ at 665 nm will appear. A check of the fluorescence can give further information whether monomeric $\mathrm{Chl}\ a \cdot H_2O$ has formed, since the aggregated $(\mathrm{Chl}\ a \cdot H_2O)_n$ is non-fluorescent, whereas the monomeric $\mathrm{Chl}\ a \cdot H_2O$ is.

tained. It is not clear, however, that the hemi-hydrate is stable, for there is some evidence to show that further pumping leads to the loss of more water. Thus, the chlorophyll *a*·monohydrate appears to be the stable chlorophyll *a* hydrate, but a hemi-hydrate of lower stability may also be formed under conditions that we have not precisely delimited. Hydration of films or evaporation from solvents in the absence of a separate water phase are therefore recommended.

Chlorophyll *b* films cast from carbon tetrachloride were hydrated by water vapor at 56° for 3 days. After 1 h of pumping at 10^{-2} Torr, products with a chlorophyll *b*–water mole ratio of 1:1.05 were obtained. This hydrate is distinctly less stable than the chlorophyll *a*·monohydrate, for after 24 h of pumping the ratio decreased to 1:0.60, probably indicating the formation of a 3:2 hydrate. We conclude that a chlorophyll *b*·monohydrate can be obtained by hydration of a film under these conditions, but that water is lost from it on pumping.

Chlorophyll *b* can also be hydrated by slow evaporation of water-saturated benzene or carbon tetrachloride solutions. The chlorophyll *b* concentration must exceed $5 \cdot 10^{-2}$ M. The evaporations were carried out in vacuum in the presence of water vapor; the resulting films were pumped on for 1 h in a 10^{-2} Torr vacuum. The product is close to chlorophyll *b*·monohydrate in composition (Table II). That this monohydrate is of different structure than that obtained by hydration of chlorophyll *b* film is shown by the infrared spectra. Both monohydrates of chlorophyll *b* yield an X-ray diffraction pattern and thus can be construed as ordered or "crystalline". The hydration properties of chlorophyll *b* are quite different from that of chlorophyll *a*, probably as a result of the differences in the nature of the C=O---Mg interactions, which appear to be stronger in chlorophyll *b*.

Pheophytin

Hydration of pheophytin *a* films or evaporation of water-saturated solutions of pheophytin in benzene or CCl_4 followed by pumping at room temperature yielded products in which the water content was 1:0.1 ± 0.1. Pheophytin films appear to be even more impervious to the passage of water than are those of chlorophyll. We believe that the very slow removal of water from pheophytin films even on heating is due to mechanical reasons. Pheophytin appears to form no stable hydrates, and its hydration and dehydration behavior are basically different from those of chlorophyll. The presence of water does not affect the carbonyl region in the infrared or the visible absorption spectra. The presence of a central magnesium atom thus appears to be a prerequisite for stable hydrate formation.

DISCUSSION

Solutions of chlorophyll in nonpolar solvents rigorously free of extraneous electron donor molecules occur as dimer (probably trimer in the case of chlorophyll *b*) or oligomers. In all of these aggregated species of chlorophyll, the force holding the dimer, trimer, or oligomer together is a coordination interaction involving the carbonyl oxygen function of one chlorophyll molecule and the central magnesium atom of another: C=O---Mg. In the case of chlorophyll *a*, it is the keto C=O oxygen that is implicated, whereas in chlorophyll *b* it is primarily the aldehyde C=O that is involved in trimer formation, whereas the keto C=O oxygen function appears to be involved in the formation of higher aggregates[9]. In the chlorophyll *a* dimer, one of the magnesium atoms still has the coordination number 4, and it is, therefore, still coordinatively unsaturated. Thus, the aggregation process can continue according to the equilibrium

$$n(\mathrm{Chl}_2) \rightleftarrows (\mathrm{Chl}_2)_n \qquad (1)$$

to form chlorophyll *a* oligomers. The value of n is essentially unity in carbon tetrachloride or benzene solutions, even at high chlorophyll concentrations, and thus chlorophyll *a* exist in these solvents almost entirely as dimer[36]. In aliphatic or cycloaliphatic solvents, such as hexane or butylcyclohexane for example, n may have a value as high as 10 to 15 in concentrated solution, hence oligomers of sizeable molecular weights are formed[36]. The keto C=O---Mg interactions forming oligomers from dimers are slightly weaker than the interaction leading to the formation of dimers; the dimers and the oligomers are clearly differentiated by their infrared spectra[9]. Nucleophiles can compete for the coordination site of magnesium. Thus, chlorophyll dissolved in electron donor solvents such as alcohols, ethers, ketones and the like will be disaggregated and will occur as chlorophyll monomers with solvent molecules in one or both axial positions. Water is a strong nucleophile for the magnesium in chlorophyll. If water is added to a benzene or carbon tetrachloride solution, the following equilibria will be obtained:

$$\mathrm{Chl}_2 + \mathrm{H_2O} \rightleftarrows \mathrm{Chl}_2\cdot\mathrm{H_2O} \qquad (2)$$

$$\mathrm{Chl}_2\cdot\mathrm{H_2O} + \mathrm{H_2O} \rightleftarrows \mathrm{Chl}_2\cdot(\mathrm{H_2O})_2 \qquad (3)$$

$$\mathrm{Chl}_2\cdot(\mathrm{H_2O})_2 \rightleftarrows 2\mathrm{Chl}\cdot\mathrm{H_2O} \qquad (4)$$

$$\mathrm{Chl}_2 + 2\mathrm{H_2O} \rightleftarrows 2\mathrm{Chl}\cdot\mathrm{H_2O} \qquad (5)$$

The concentration of chlorophyll is an important factor in these equilibria because the solubility of water is limited to $8\cdot10^{-3}$ M in carbon tetrachloride and 10^{-2} M in benzene. A very dilute chlorophyll solution in water-saturated carbon tetrachloride or benzene will therefore exist as the chlorophyll monomer·monohydrate. In concentrated chlorophyll solution, the chlorophyll will occur as a mixture of chlorophyll·dimer·monohydrate and chlorophyll·monomer·monohydrate. The dimer·dihydrate according to equilibrium (Eqn. 3) we consider of only minor importance because of the reluctance of magnesium to adopt a coordination number of 6, which it appears to do only under forcing conditions if at all. The dimer·dihydrate must, therefore, disproportionate into monomer·monohydrate. When a carbon tetrachloride solution of chlorophyll is evaporated in the presence of excess water vapor, under the conditions that we describe, the monohydrate is the stable species. Conversely, because both benzene and CCl_4 form azeotropes with water, repeated evaporation with these solvents will displace the equilibria in the direction of dimer, and anhydrous chlorophyll will be produced.

In forming the hydrate, a C=O---Mg interaction is broken and a Mg---OH_2 interaction replaces it. These are both quite strong interactions, but the difference in energy between them is probably small. Conversely, the removal of water from a CCl_4 or benzene solution is compensated for by the reformation of C=O---Mg interactions. In a film or solid, the lack of mobility of the chlorophyll molecule makes it difficult to reform C=O---Mg interactions as water is removed from a hydrated film or solid, because only a small fraction of the chlorophyll molecules will be oriented so that carbonyl interactions can replace the Mg---OH_2. It is this factor to which we attribute the stability of ($Chl \cdot H_2O$) in films or solids.

There is another aspect to the interaction of water and chlorophyll that requires special note. In aliphatic or cycloaliphatic solvents, another equilibrium comes into play:

$$n(Chl \cdot H_2O) \rightleftarrows (Chl \cdot H_2O)_n \qquad (6)$$

In carbon tetrachloride or benzene, this equilibrium is displaced strongly to the left. In aliphatic hydrocarbon solvents, for reasons associated most likely with the solvation characteristics of this class of solvents, equilibrium (Eqn. 6) is displaced far to the right. Water coordinated to the magnesium atom of one chlorophyll molecule forms hydrogen bonds to the keto and carbomethoxy carbonyl oxygen functions of another chlorophyll molecule, and repetition forms a large chlorophyll–water adduct or micelle of colloidal dimensions. The stoichiometry of this species is 1:1. This species will form in carbon tetrachloride only under conditions of very high chlorophyll concentration, and this is the rationale of the evaporation procedure we describe for hydrate preparation.

It is possible under certain conditions to obtain the hemihydrate. We believe it should be represented as $Chl \cdot Chl \cdot H_2O$ rather than $Chl \cdot H_2O \cdot Chl$. Whereas solid chlorophyll *a*·monohydrate is yellow-green and can easily be identified as hydrated by visual examination, the blue $Chl_2 \cdot H_2O$ is indistinguishable in color from anhydrous chlorophyll. Although in terms of weight, the amount of water present in the monohydrate is small, profound changes in the X-ray diffraction patterns, infrared and visible absorption spectra are observed when this water is lost from $Chl \cdot H_2O$.

The situation for chlorophyll *b* is considerably more complex. In carbon tetrachloride solution, chlorophyll *b* appears to consist of the trimer; it is not settled at this time whether our earlier report that chlorophyll *b* occurs as dimer in chloroform[2] is an error or reflects a real difference in the solvents. The occurence of chlorophyll *b* as trimer in CCl_4 would account for a 3:2 ($H_2O(Chl_3)H_2O$) or 3:1 ($H_2O(Chl_3)$) hydrate. The aldehyde C=O---Mg interaction appears considerably stronger than the keto C=O---Mg interaction and is not as readily ruptured by water.

Under forcing conditions of high water activity, however, hydration of chlorophyll *b* either as a film or in concentrated carbon tetrachloride solution ($5 \cdot 10^{-2}$ M) leads in both cases to a chlorophyll *b* monohydrate. The infrared spectrum of both chlorophyll *b* monohydrates shows in the C=O region basically the same feature, namely, absence of the C=O absorption at 1700 cm^{-1} (assigned to the uncoordinated keto carbonyl) and appearance of the band at 1645 cm^{-1} (assigned to a C=O-H_2O-Mg interaction)[9]. However, a band at 1670 cm^{-1} noted for the chlorophyll *b* hydrated as a film appears at 1680 cm^{-1} for the chlorophyll *b* hydrated in carbon tetrachloride solution. This we taken as indicative of some difference in structure between the two monohydrates. Nevertheless, both monohydrates of chlorophyll *b* appear to give essentially the same X-ray powder pattern*.

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* In the literature are reports[16,37] that chlorophyll *b* fails to give an X-ray diffraction pattern under conditions of preparation which lead to "crystalline" chlorophyll *a*. The reason for this is now clear. The (aggregated) chlorophyll *b* monohydrate can only be obtained under very particular conditions, and even then two modifications are possible, of which only one is reasonably stable. It may be worth noting that the powder patterns of chlorophyll *a* and chlorophyll *b* monohydrates are observed to be basically different. The spacings for both chlorophyll *b*·monohydrates are found to be 10.0 Å (strong), 4.45 Å (diffuse), 3.47 Å (strong), 2.7 Å (very weak). This would seem to indicate that in both structures the orientation of the magnesium atoms at least is very similar.

Both chlorophyll *b*·monohydrates have a characteristic olive-green appearance, which serves to differentiate them from the green of the lower hydrates or anhydrous chlorophyll *b* itself.

Hydration in solution will reflect the kind and amount of the various chlorophyll dimers, trimers, oligomers, and chlorophyll–water micelles that are in equilibrium. This in turn depends on the kind of nonpolar solvent, the chlorophyll concentration, the temperature, and the thermodynamic activity of the water. Evaporation of such solutions will generally lead to some water loss by azeotrope formation, and thus it is not too surprising that small deviations in the stoichiometry of the hydration products are encountered.

The sensitivity of hydration to the exact conditions of preparation is worth pursuing. We have shown by infrared studies[38] that at a reduced water vapor pressure (6–8 mm Hg) a chlorophyll *a*–water interaction occurs that does not interrupt the C=O/Mg coordination of the chlorophyll *a* oligomers. The infrared data are best explained by the assumption that the water is hydrogen bonded to the free keto-carbonyl and the ester-carbonyl functions. Though it appears unlikely that this mode of hydration is stoichiometric, model studies show that at least in solutions the chlorophyll oligomers structure would allow a specific ester $C{=}O/H\diagdown_{O}\diagup H/O{=}C$ ester interaction, either within the same chlorophyll molecule or between adjacent chlorophylls in the oligomer. This water is only weakly bonded, and it can be easily pumped off at room temperature. Nevertheless, the presence of water bound in this way affects the red band of the absorption spectrum of chlorophyll *a* in the visible region.

The state of aggregation of chlorophyll in nonpolar solvents may be strongly

TABLE III

ABSORPTION SPECTRA OF CHLOROPHYLLS IN VARIOUS STATES OF HYDRATION IN THE 300–800-nm REGION IN THE VISIBLE IN FILMS AND IN CYCLO-ALIPHATIC HYDROCARBON SOLUTIONS

Compound	*State of hydration (chlorophyll/H_2O, mole ratio)*	*Soret band*		*Red band*	
		*Film**	*Solution***	*Film**	*Solution***
Chlorophyll *a*	Anhydrous	436	430	674	664, 675 (shoulder)
	1:1	448	448	738–757***	742–747
Chlorophyll *b*	Anhydrous	462	452	652	643, 660
	3:1, 3:2	465 §	452	654 §	643
	1:1	470	452, 470 (shoulder)	687	642, 690
Pheophytin *a*	Anhydrous	421, 440	411	695	670
	"Hydrated"	421, 440	411	695	670

* Cast from cyclohexane.

** About $3 \cdot 10^{-4}$ M solution in cyclohexane.

*** Maximum depends on which solvent is used to cast the film prior to hydration. If the chlorophyll *a* is hydrated in solution, a film cast from cyclohexane has its absorption maximum at 742 nm.

§ Cast from carbon tetrachloride.

TABLE IV

INFRARED ABSORPTION SPECTRA OF CHLOROPHYLL FILMS* IN VARIOUS STATES OF HYDRATION IN THE 1800–1600-cm^{-1} REGION

vs = very strong; s = strong; m = medium; w = weak; vw = very weak; sh = shoulder.

Compound	*State of hydration (chlorophyll/H_2O mole ratio)*	*1800–1600* cm^{-1}				
Chlorophyll *a*	Anhydrous	1737(s)	1695(m)		1655(s)	1610(m)
	1:1	1738(s)	1695(vw)		1640(vs)	1605(w)
Chlorophyll *b*	Anhydrous	1736(s)	1698(s)		1663(m)	1606(m)
	3:1, 3:2	1736(s)	1698(s)		1663(m)	1606(m)
	1:1**	1737(s)	1680(s)		1645(vs)	1612(m)
	1:1***	1737(s)	1700(sh)	1670(s)	1645(s)	1612(m)
Pheophytin *a*	Anhydrous	1740(s)	1700(s)			1622(m)
	"Hydrated"	1740(s)	1700(s)			1622(m)

* Films cast from cyclohexane on IRTRAN-2 plates. Assignments are to be found in ref. 9.

** Hydrated as $>5 \cdot 10^{-2}$ M solution in carbon tetrachloride.

*** Hydrated as film at 55°.

affected by the presence of water, hence the infrared and visible absorption spectra are likewise water-dependent. Consequently, the presence or absence of water in chlorophyll solutions can be established at least approximately from infrared and absorption spectra in the visible. Because instruments for these measurements are widely available, we have recorded the visible absorption (Table III) and infrared (Table IV) spectra for chlorophyll films and solutions containing known amounts of water, as established by vapor phase chromatography. Although the infrared and visible spectra are not as quantitative a measure of water content as is direct vapor phase chromatographic analysis, nevertheless, the data in Tables III and IV are useful for orientation purposes and are sufficient to establish composition in many experimental situations involving the chlorophylls. We believe that judiciously used these tables may be of help to minimize some of the problems resulting from the unique nature of the chlorophyll–water interaction.

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