An Infrared Study of Chlorophyll-Chlorophyll and Chlorophyll-Water Interactions[1a]

Karlheinz Ballschmiter[1b] and Joseph J. Katz

Contribution from the Chemistry Division, Argonne National Laboratory, Argonne, Illinois 60439. *Received September 25, 1968*

Abstract: The infrared spectra of chlorophyll a, chlorophyll b, bacteriochlorophyll, and pyrochlorophyll a in nonpolar solvents in the 1600–1800- and 3000–3800-cm^{-1} regions have been studied in the presence and absence of water. The data in dry systems are interpreted in terms of chlorophyll–chlorophyll dimers and oligomers formed by coordination interaction between the C-9 ketone oxygen function of one chlorophyll molecule and the central magnesium atom of another: keto C=O---Mg. In systems containing water, the results of chlorophyll–water interaction are found to be solvent and concentration dependent. In CCl_4 or benzene at chlorophyll concentrations of 10^{-6} to 10^{-2} *M* and in aliphatic hydrocarbon solvents at chlorophyll concentrations of 10^{-6} to 10^{-5} *M*, water acts as a nucleophile to form $Chl_2 \cdot H_2O$ and $Chl \cdot H_2O$ species. In aliphatic or cycloaliphatic hydrocarbon solvents at chlorophyll concentrations in the range 10^{-4} to 10^{-2} *M*, water coordinated to the Mg of one chlorophyll molecule is hydrogen bonded to the ketone and carbomethoxy carbonyl functions of another chlorophyll molecule: keto C=O---HO(Mg)H---O=C ester. Repetition of this interaction results in the formation of chlorophyll–water micelles of colloidal dimensions. A set of equilibria is advanced to account for the effects of solvent, concentration, and temperature on the chlorophyll species present in solution. The ir spectra are used to suggest structures for the dimers, oligomers, and chlorophyll–water adducts.

The structural formula of chlorophyll as usually written (Figure 1) shows the central magnesium atom with coordination number 4. Recent infrared[2-5] and nuclear magnetic resonance studies,[6,7] however, have provided evidence that the coordination number of magnesium in chlorophyll is always larger than 4, and that the coordination properties of magnesium are decisive among the factors that determine the state of chlorophyll in solution. In electron-donor solvents, the coordination unsaturation of the magnesium is relieved by the solvent molecules. In solvents such as pyridine, tetrahydrofuran, acetone, and the like, chlorophyll is monomeric, with solvent molecules in the axial position(s). In nonpolar solvents, the coordination unsaturation of the magnesium cannot be satisfied by the solvent, and coordination interaction of the ketone oxygen function of one chlorophyll molecule with the magnesium of another is the option that is exercised. Thus, in nonpolar solvents, chlorophyll occurs as dimers or oligomers held together by C=O---Mg interactions. Nmr and ir studies have been useful in establishing the geometry of the dimers and in clarifying the participation of the ketone oxygen function at C-9 in the self-aggregation of chlorophyll.[8,9]

Nucleophiles (alcohols, ethers, ketones, esters, and amines) can compete for coordination to the magnesium of chlorophyll dissolved in nonpolar solvents. Extraneous nucleophiles disaggregate chlorophyll–chlorophyll dimers with the formation of chlorophyll monomer monosolvates. A detailed examination of chlorophyll–alcohol interactions by nmr has recently been made.[7] Other studies indicate that carotenoids containing hydroxyl or epoxide groups coordinate to the magnesium of chlorophyll,[9] whereas plant sulfolipid[9] and quinones[9] interact weakly by coordination if at all in CCl_4 solution.

The nucleophile that is by all odds the most interesting in the present context is water. The photolysis of water is the central event of photosynthesis, and there is every reason to suppose that chlorophyll is involved in this process. Thus, Witt and coworkers[10] found that a long-wavelength form of chlorophyll, chlorophyll a_{11}-435–682, is involved in the photolysis of water. The nature and consequences of the nucleophilic interaction between water and chlorophyll, and whether water acting as a nucleophile for chlorophyll has any unique properties of importance to photosynthesis, thus become questions of considerable interest.

Water, it has long been surmised, has a special relation to chlorophyll.[11] Willstätter and Stoll[12] on the basis of combustion data on a mixture of chlorophylls a and b decided that chlorophyll and the alkyl chlorophyllides contained 0.5 mol of water/mol of chlorophyll. The hydration conditions were not described and the experimental basis for this widely quoted conclusion is extremely tenuous.[13] Chlorophyll and water have been reported[11,14] to constitute a nonstoichiometric system whose composition is determined by the partial pressure

(1) (a) Based on work performed under the auspices of the United States Atomic Energy Commission; (b) Resident Research Associate, 1967–1969; University of Mainz, Germany.

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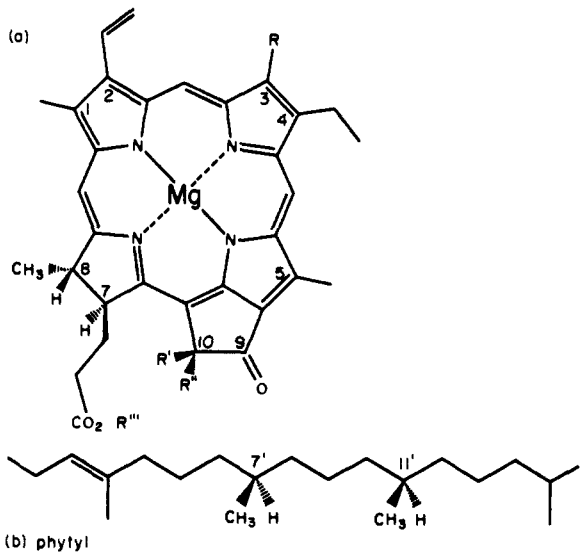

Figure 1. Structural formulas of compounds. In the Mg-free compounds, the magnesium is replaced by 2H. In bacteriochlorophyll, the vinyl group at position 2 is replaced by an acetyl group, and two additional H's are present at positions 3 and 4.

	Mg Present	R	R′	R″	R‴
Chlorophyll a	+	CH_3	H	CO_2CH_3	Phytyl
Chlorophyll b	+	CHO	H	CO_2CH_3	Phytyl
Methyl chlorophyllide a	+	CH_3	H	CO_2CH_3	CH_3
Pyrochlorophyll a	+	CH_3	H	H	Phytyl
Pheophytin a	−	CH_3	H	CO_2CH_3	Phytyl
Pyropheophytin a	−	CH_3	H	H	Phytyl
Bacteriochlorophyll	+	CH_3	H	CO_2CH_3	Phytyl

of water. Jacobs, Vatter, and Holt[15] reported that water was essential for the preparation of "crystalline" chlorophyll, and it is this work that is primarily responsible for the wide-spread conviction that chlorophyll–water interactions have some special structural significance. Recently, Sherman and Wang[16] made detailed infrared observation on wet and dry chlorophyll a. The most important observation they made was that the decrease of the free keto C=O absorption peak at 1698 cm^{-1} and the increase in the peak near 1650 cm^{-1}, sometimes seen in chlorophyll solutions and previously attributed to higher states of chlorophyll aggregation,[8] in some unspecified way involved water. However, Sherman and Wang[16] were unable to reconcile their results (obtained in Nujol "mulls") with the ir data on chlorophyll already in the literature, for the most part obtained on CCl_4 and $CHCl_3$ solutions, and were unable to interpret either their infrared or visible absorption spectra.

An informative investigation by infrared spectroscopy on the effects of water on the state of aggregation of chlorophyll a in carbon tetrachloride solution was made by Rappaport and Van Winkle.[17] Rappaport found that water acts as a nucleophile for chlorophyll a in CCl_4 solution, interacting according to the equation $Chl_2 + 2HOH \rightleftharpoons 2Chl \cdot H_2O$. Because Rappaport studied the effect of water only in CCl_4 solutions, he observed only a part of the chlorophyll a–water interactions described here.

Some aspects of the work described here have appeared elsewhere in preliminary form.[18,19] Here, we describe in detail the infrared spectra of dry and hydrated chlorophyll a, chlorophyll b, bacteriochlorophyll, and pyrochlorophyll a[20] in various nonpolar solvents. The ir observations are best interpreted by a set of equilibria involving chlorophyll–chlorophyll interaction products, chlorophyll monomer monosolvates, and chlorophyll–water adducts, $(Chl \cdot H_2O)_n$, of colloidal dimensions.

Experimental Section

Chlorophylls. These were prepared by the usual procedures.[21]

Ir Spectra. Ultramicro Beckman KBr cells (0.025–1.0 mm) were used for the carbonyl region spectra. No changes in the spectra of the hydrated chlorophylls could be observed when the samples were allowed to stand in the cells 24 hr. For the water region, quartz cells of 10-mm path lengths were used because of the better presentation of the 3000–3800-cm^{-1} region. Spectra were recorded with a Beckman IR-7 spectrometer, generally with a beam condenser. A screen was used in the reference beam, but in some experiments a variable path length cell containing the solvent was placed in the reference beam when compensation for the solvent peaks was required. The spectra are highly reproducible, and all of the spectra shown in this paper have been replicated. Although the peaks are broad, the maxima are quite reproducible (± 1 cm^{-1}).

Solvents. The best reagent grade benzene, cyclohexane, *n*-butylcyclohexane, dodecane, and hexadecane were intensively dried over calcium hydride. Carbon tetrachloride was dried over silica gel. Solvents and solutions were handled in a drybox in an N_2 atmosphere or on the vacuum line with minimal exposure to the atmosphere. The water content of all solvents was checked by gas phase chromatography (see next section), ir in the OH stretch region, or by Karl Fischer titration.

Results

Hydration of Chlorophylls. As the water content of the systems we will describe is critical, we will discuss the hydration of chlorophyll and the analytical problems involved in determining the chlorophyll–water ratio in detail. To study chlorophyll–water interactions it is obviously desirable to start with truly anhydrous chlorophyll, to specify the composition of chlorophyll hydrates used for making up solutions, and to determine the water content of chlorophyll solutions. We have therefore developed a vapor-phase chromatographic procedure for the analysis of water in chlorophyll solutions. We use a 35-cm, 4-mm i.d. stainless steel column, packed with uncoated Poropak Q for the separation, and a tungsten WX thermal conductivity cell for the detection of the water. Hydrated chlorophyll (solid or film) is dissolved in dry benzene containing dry acetone as internal standard and an aliquot large enough to contain 1 to 10 μg of H_2O injected into a Varian Aerograph preparative chromatograph, Model A-700. The injection port is at 180°, and the column is at 110°. We can detect 0.1 to 0.2 μg of water and can readily measure 5 μg of water

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in 50 μl of solution with better than 10% precision. Details of the procedure will be given elsewhere.[13]

A second procedure we have used for the semiquantitative determination of the water content of chlorophyll solutions is the measurement of the intensity of the OH absorption of monomeric water in the ir fundamental region at 3706 cm^{-1}. This procedure is similar to the one reported by Rappaport.[17] By using concentrated chlorophyll solutions and 10-mm cells, the absence of absorption in the OH stretch region can be shown to indicate a water content in the solution less than 10^{-5} *M*. This procedure is best used to demonstrate the absence of water. Any solution we describe as dry contains less than 10^{-5} *M* water. For quantitative determination of the water content of hydrated films or chlorophyll solutions, we find the vapor phase chromatographic procedure the better choice.

Finally, the ir spectra in the carbonyl region, the principal object of our investigations, are themselves accurate guides to the water content of the solutions. The absence of a 1638-cm^{-1} absorption peak, together with the absence of absorption in the OH stretch region, is confirmed by vpc to indicate a mole ratio of chlorophyll to water of at least 100:1.

We have established conditions whereby chlorophylls a and b, bacteriochlorophyll, and pyrochlorophyll a can be rendered anhydrous. Chlorophyll a is dissolved in dry CCl_4 and the solvent removed by evaporation at room temperature in a stream of dry nitrogen. Repeating this procedure three times results in a product with a chlorophyll-to-water ratio greater than 10:1. When chlorophyll dried by codistillation with CCl_4 is heated under vacuum (10^{-5} mm) for 30 min at 60–70°, vpc shows the chlorophyll–water mole ratio as an upper limit to be at least 100:1. Very small amounts of water are involved as it is obvious from the relative molecular weights of chlorophyll and water, and it is very difficult, even when precautions are taken, to avoid the introduction of some water during analysis. Thus, the water content of dry chlorophyll given here is quite conservative and constitutes an upper limit. In the ir, the dehydrated chlorophyll a (10^{-2} *M* in CCl_4) shows no OH absorption in a 10 mm cell, indicating less than 10^{-5} *M* water to be present. We have also established that heating chlorophyll a for 4 hr at 80° under a 10^{-5} mm vacuum causes no degradation or change in the chlorophyll, as shown by column chromatography on powdered sugar.

Chlorophyll b is more difficult to dehydrate than is chlorophyll a. The stable hydrate of chlorophyll b appears to be the trimer monohydrate or the trimer dihydrate.[13] Evaporation of a solution of chlorophyll b in CCl_4 in a stream of dry N_2, a procedure that gives an essentially anhydrous chlorophyll a, results in chlorophyll b which still has a broad absorption band in the OH region. However, chlorophyll b can be obtained in anhydrous form by dissolving the b in benzene, and distilling the benzene away under N_2 at 1 atm pressure, at 60°. These procedures, codistillation of water with benzene or CCl_4, at elevated temperatures and atmospheric pressure followed by heating under high vacuum, yield anhydrous chlorophylls a and b, bacteriochlorophyll, and pyrochlorophyll a suitable as starting materials for the studies reported here.

We have approached the problem of preparing solutions containing known amounts of water by finding conditions for the preparation of chlorophyll hydrates of known stoichiometry. A film of dry chlorophyll a exposed to water vapor at 50° for 24–48 hr, and then pumped on for 1 hr at 23° under a vacuum of 10^{-2} mm, has the composition chlorophyll·H_2O according to vpc analysis.[13] Chlorophyll a prepared by standard procedures involving contact with water during preparation or exposed to laboratory air also is found to be the monohydrate. Chlorophyll a monohydrate in the ir shows (in *n*-butylcyclohexane solution) strong ester splitting, practically no free C=O absorption at 1695 cm^{-1}, and a strong absorption maximum at 1638 cm^{-1}. Solutions of chlorophyll a·H_2O in aliphatic solvents are yellow green in color, with an absorption maximum in the visible at 740–745 nm. In our experience, absence of the absorption maximum at 740–745 nm in aliphatic hydrocarbon solutions is an excellent criterion for the absence of water. Conversely, if the 665–673-nm absorption maximum is mostly absent, and only the 740–745-nm absorption peak is observed, the magnesium in the chlorophyll a is maximally hydrated (chlorophyll–water, 1:1).

To obtain hydrated chlorophylls we have used the following procedures. (1) Chlorophylls a, b, or bacteriochlorophyll were dissolved in cyclohexane by warming to 50° to form a 10^{-3} *M* solution. Water (10 μl) was added to 2–3 ml of solution and sonicated (Macrosonics Corp.) until all the water was dispersed. The solution was again warmed to 50° and the warm solution sonicated again; for chlorophyll b and bacteriochlorophyll warming and sonication were repeated at least three times. Then the cyclohexane was removed by evaporation in a stream of nitrogen. Chlorophylls a and b, and bacteriochlorophyll hydrated this way show no free keto C=O absorption in the ir, and can be considered to be the at least 1:1 hydrates. (2) Anhydrous chlorophylls a or b were dissolved in water-saturated CCl_4 or benzene, and the solvent was evaporated slowly (24–48 hr) in a water-saturated N_2 atmosphere (100 mm). Superficial excess water was then removed by pumping for 1 hr at 10^{-2} mm at room temperature. The chlorophylls so obtained are the 1:1 monohydrates as determined by direct water analysis. Dry chlorophyll solutions can also be hydrated by exposure to water vapor under vacuum. The vapor pressure of the water vapor must be controlled, and the solvent used may affect the degree of hydration achieved.

Chlorophyll–Chlorophyll Interactions. In nonpolar solvents, the coordination unsaturation of the central magnesium atom in chlorophyll is satisfied by the ketone oxygen function of another chlorophyll molecule, resulting in the formation of dimers and higher aggregates *via* C=O---Mg bonding. This is the important mode of interaction in the absence of extraneous nucleophiles. The extent to which coordination interactions lead to the further formation of oligomers depends on a variety of factors, one of which is the solvation characteristics of the nonpolar solvent used. A close examination of the ir spectra reveals much more about the nature of the chlorophyll interactions. The assignments of the ir spectra offered here follow closely the earlier assignments in the prior literature.[8]

Ir spectra in the carbonyl region for dry chlorophylls a, b, bacteriochlorophyll, and pyrochlorophyll a in dry

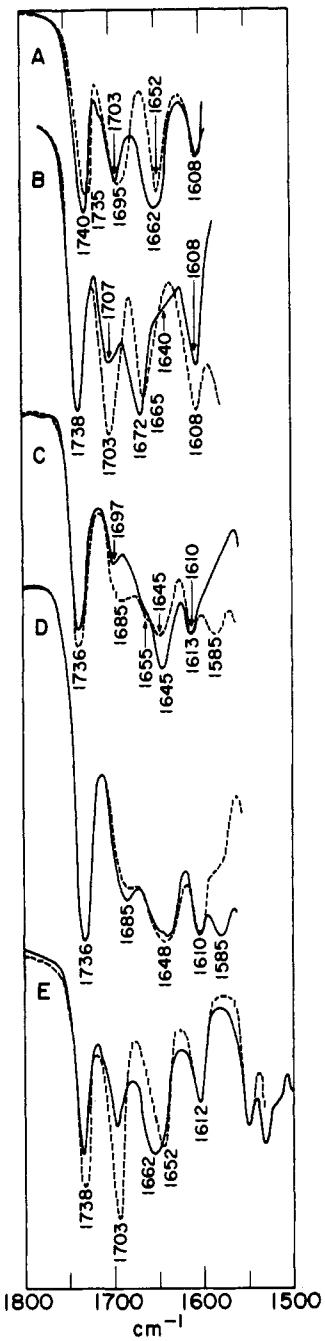


Figure 2. Ir spectra of anhydrous chlorophylls in the 1800–1600-cm^{-1} region in CCl_4 and *n*-butylcyclohexane (NBC) solutions: A, chlorophyll a, (– – –) in CCl_4 (2.0×10^{-2} *M*, 0.15 mm cell), (——) in NBC (3.2×10^{-2} *M*, 0.15 mm cell); B, chlorophyll b, (– – –) in CCl_4 (2.0×10^{-2} *M*, 0.2 mm cell), (——) in NBC (2.0×10^{-2} *M*, 0.2 mm cell); C, bacteriochlorophyll, (– – –) in CCl_4 (2.3×10^{-2} *M*, 0.2 mm cell), (——) in NBC (2.1×10^{-2} *M*, 0.2 mm cell); D, bacteriochlorophyll, (– – –) in CCl_4 (3.2×10^{-2} *M*, 0.2 mm cell), (——) in CCl_4 (2.5×10^{-1} *M*, 0.025 mm cell); E, chlorophyll a, (– – –) in NBC (1.5×10^{-3} *M*, 1.77 mm cell; solvent compensation in reference beam), (——) in NBC (2.6×10^{-2} *M*, 0.1 mm cell).

carbon tetrachloride and in *n*-butylcyclohexane solution are shown in Figure 2. *n*-Butylcyclohexane may be taken as more or less typical of alicyclic hydrocarbon solvents; very similar spectra are observed in cyclohexane, dodecane, hexadecane, or alkylcyclohexane solutions. Chlorophyll a (Figure 2A) in CCl_4 solution shows ester absorptions at 1735 cm^{-1}, free keto carbonyl at 1695 cm^{-1}, coordinated keto C=O---Mg at 1652 cm^{-1}, and skeletal C=C, C=N absorption maxima at 1608 cm^{-1}. A comparable solution of chlorophyll a in *n*-butylcyclohexane shows the ester and skeletal absorption maxima nearly unchanged. The free keto C=O peak (at 1703 cm^{-1}) is decreased, and the keto C=O---Mg aggregation peak in solution more concentrated than 5×10^{-3} *M* is shifted to *shorter* wavelength (1662 cm^{-1}). That is, the average shift to lower frequencies in the ketone C=O absorption due to coordination is less. In CCl_4 or in *n*-butylcyclohexane solutions less concentrated than 10^{-3} *M*, the 1652-cm^{-1} peak may be taken to indicate the presence of chlorophyll a dimers.[2] The diminution of the free keto peak in the *n*-butylcyclohexane solution in solutions more concentrated than 10^{-3} *M* and the occurrence of the maximum at shorter wavelengths (1662 cm^{-1}) we attribute to the formation of higher chlorophyll oligomers, formed by chlorophyll a dimers as the repeating unit. *n*-Butylcyclohexane is a distinctly more aggregating solvent for dry chlorophyll a than is CCl_4. The shift of the aggregation peak to 1662 cm^{-1} can be construed to show the presence of two energetically different C=O---Mg interactions. In a 0.12 *M* chlorophyll a solution in *n*-hexane, the average composition is found to be $(Chl_2)_9$ by vapor phase osmometry.[22]

The differences between chlorophyll b spectra in CCl_4 and in *n*-butylcyclohexane solution are much more pronounced than is the case for chlorophyll a (Figure 2B), and the spectra indicate that *n*-butylcyclohexane is a much less favorable solvent for chlorophyll b than it is for chlorophyll a. The free keto carbonyl absorption maximum at 1703 cm^{-1} in CCl_4 is strongly diminished in *n*-butylcyclohexane, and at the same time, the keto C=O---Mg aggregation peak at 1665 cm^{-1} becomes more intense and is shifted to higher frequencies, now appearing at 1672 cm^{-1}. The free aldehyde C=O, which contributes to the 1665-cm^{-1} absorption peak, likewise decreases in intensity. The 1608-cm^{-1} skeletal peak, which includes a contribution from aldehyde C=O---Mg interactions, also becomes more intense. We judge from the spectra that the increase in the degree of aggregation of chlorophyll b in *n*-butylcyclohexane involves keto interactions with magnesium to a much greater extent than it does the aldehyde C=O. Such a conclusion is consistent with the existence of chlorophyll b in CCl_4 as a trimer,[2] a structure in which the aldehyde C=O is already fully employed; further aggregation must then occur at the expense of the remaining free keto C=O in the trimer. As in the case of chlorophyll a, the ester and skeletal absorption maxima are relatively unaffected by the higher state of aggregation of chlorophyll b in *n*-butylcyclohexane solution.

In CCl_4 solutions of bacteriochlorophyll (Figure 2C), the ester absorption peaks are at 1736 cm^{-1}, free keto C=O at 1685 cm^{-1}, free acetyl C=O at 1655 cm^{-1}, keto C=O---Mg at 1645 cm^{-1}, and skeletal vibrations plus acetyl C=O---Mg at 1610 cm^{-1}. In *n*-butylcyclohexane solution, the free keto C=O is strongly diminished (1697 cm^{-1}), and the keto aggregation peak at 1645 cm^{-1} is strongly enhanced. From the persistence

(22) K. Ballschmiter, K. Truesdall, and J. J. Katz, manuscript in preparation.

of the shoulder at 1660 cm^{-1}, and the invariance of the peak at 1613 cm^{-1}, it appears that the increase in the state of aggregation of bacteriochlorophyll in *n*-butylcyclohexane is mainly at the expense of free keto C=O. Here also, it is evident that *n*-butylcyclohexane is a far less favorable solvent for bacteriochlorophyll than it is for chlorophyll a.

It has been shown by Rappaport[17] that the ir spectra of CCl_4 solutions of chlorophyll a are almost independent of concentration over a 200-fold range of concentration. That the state of aggregation is relatively independent of concentration in CCl_4 is also true for bacteriochlorophyll (Figure 2D). Over a tenfold concentration range, the ir spectrum shows only a small decrease in the free keto peak at 1685 cm^{-1} and only a small shift to higher frequencies in the keto C=O---Mg absorption (1648 cm^{-1}). However, concentration effects on the state of aggregation are much more prominent in hydrocarbon solvents. In Figure 2E, in *n*-butylcyclohexane solution a tenfold change in chlorophyll a concentration is seen to cause the free keto C=O absorption at 1703 cm^{-1} to almost vanish, with a concomitant increase and shift of the keto C=O---Mg absorption maximum to 1662 cm^{-1}. Chlorophyll a thus appears to be considerably more aggregated in aliphatic hydrocarbons than in CCl_4 solutions. For chlorophyll b, carbon tetrachloride is already more of an aggregating solvent than it is for chlorophyll a.

Disaggregation of Chlorophyll–Chlorophyll Oligomers by Nucleophiles. Electron-donor substances added to solutions of chlorophyll oligomers in nonpolar solvents compete for coordination to magnesium, resulting in disruption of the C=O---Mg interactions with formation of monomeric chlorophyll–ligand species. This process can readily be followed by ir observations in the carbonyl region. As the chlorophyll–chlorophyll oligomers disaggregate, the free keto C=O absorption peak increases, and the keto C=O---Mg aggregation peak decreases in intensity and area. Figure 3A shows the result of added methanol to a CCl_4 solution of chlorophyll a. The aggregation peak at 1652 cm^{-1} becomes smaller and finally disappears, whereas the free keto C=O absorption at 1693 cm^{-1} increases. The ester peak at 1735 cm^{-1} present in the dimer is split into two by hydrogen bonding to the methanol; the carbomethoxy C=O peak is shifted to 1715 cm^{-1}, with the result that the ester peak appears strongly diminished in the disaggregated and hydrogen-bonded monomeric chlorophyll a. The nucleophilic behavior of methanol is complicated by its ability to form hydrogen bonds with the several carbonyl groups of the chlorophyll. It is therefore instructive to examine the behavior of nucleophiles that cannot form hydrogen bonds with the carbonyl functions of the chlorophyll. Figure 3B shows the results of a titration of a CCl_4 solution of chlorophyll a with tetrahydrofuran. The aggregation C=O---Mg peak at 1652 cm^{-1} disappears, and the absorption peak characteristic of free keto C=O appears. No ester splitting is observed with tetrahydrofuran. It is also evident that the gain in area of the free keto C=O absorption with disaggregation is much smaller than the area associated with the aggregation peak at 1652 cm^{-1} which is lost. This dictates caution in the direct use of the peak areas in deducing equilibrium constants. The extinction coefficients of the free keto C=O group in the monomer

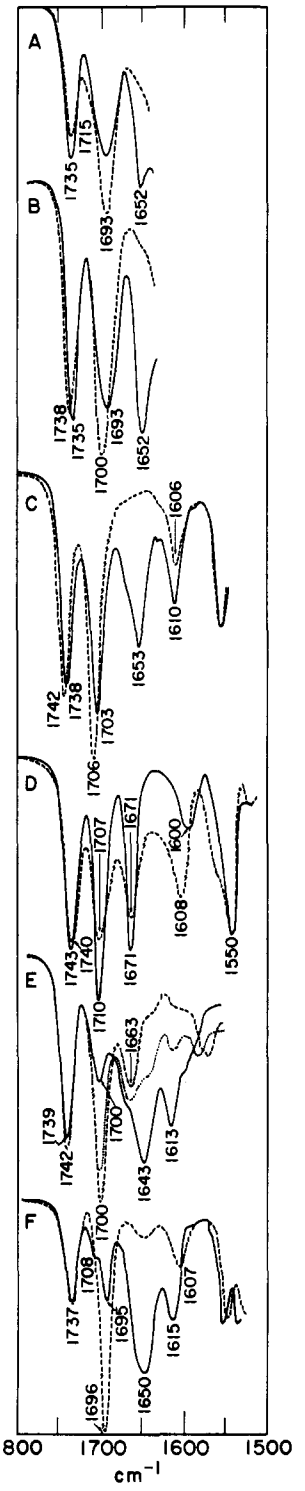


Figure 3. Disaggregation of chlorophylls from changes in ir spectra (1800–1500-cm^{-1} region) on titration with nucleophiles: A, methanol titration, 0.556 × 10^{-3} *M* chlorophyll a in CCl_4 (3 mm cell), (——) no CH_3OH, (– – –) 1.6 × 10^{-3} *M* CH_3OH; B, tetrahydrofuran (THF) titration, 1.61 × 10^{-3} *M* chlorophyll a in CCl_4 (2.5 mm cell), (——) no THF, (– – –) 3.5 × 10^{-3} *M* THF; C, THF titration, 1.9 × 10^{-3} *M* chlorophyll a in *n*-butylcyclohexane (NBC), (1.77 mm cell, solvent compensation), (——) no THF, (– – –) 3.5 × 10^{-2} *M* THF; D, THF titration of chlorophyll b, 2.20 × 10^{-3} *M* in NBC (1.77 mm cell, solvent compensation), (——) no THF, (– – –) 1.7 × 10^{-2} *M* THF; E, THF titration of bacteriochlorophyll, 1.72 × 10^{-3} *M* in NBC (1.77 mm cell, solvent compensation), (——) no THF, (···) 1.5 × 10^{-2} *M* THF, (– – –) 3.2 × 10^{-2} *M* THF; F, THF titration of pyrochlorophyll a, 2.04 × 10^{-3} *M* in NBC (1.77 mm cell, solvent compensation), (——) no THF, (– – –) 4.7 × 10^{-2} *M* THF.

Table I. Equilibrium Constants for Chlorophyll–Ligand Interactions from Ir Titration Data

$$Chl_2 + 2L \overset{K_1}{\rightleftharpoons} 2Chl \cdot L$$

Compound	Solvent	Concn of Chl, $M \times 10^3$	Ligand[b]	Concn of ligand,[d] $M \times 10^3$	K_1, mol $l.^{-1}$
Chlorophyll a	CCl_4	0.556	CH_3OH	1.6	45
Chlorophyll a	CCl_4	1.61	THF[c]	3.5	22
Chlorophyll a	NBC[a]	1.90	THF[c]	35	0.3
Chlorophyll b	NBC[a]	2.20	THF[c]	17	...
Bacteriochlorophyll	NBC[a]	1.72	THF[c]	32	...
Pyrochlorophyll a	NBC[a]	2.04	THF[c]	47	0.03

[a] NBC = *n*-butylcyclohexane; solvent compensation in reference beam. [b] Ligand was added from a 10-μl Hamilton syringe. Change in concentration from the addition of ligand was less than 5% for the maximum addition. [c] THF = tetrahydrofuran. [d] Ligand concentration at complete disaggregation of C=O/Mg interactions.

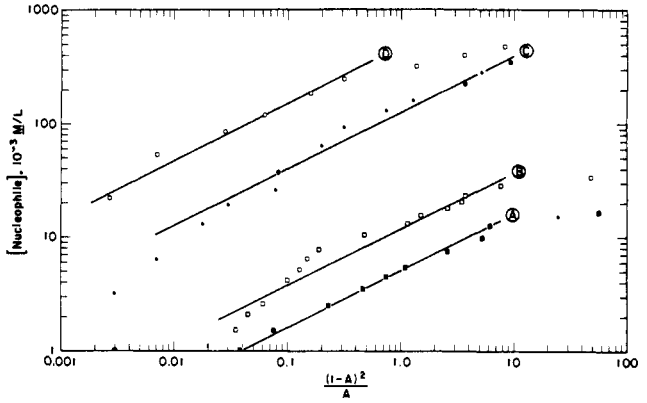


Figure 4. Chlorophyll–nucleophile interactions. For the reaction $Chl_2 + 2B \rightleftharpoons 2Chl \cdot B$, the equilibrium constant K is given by $\log C_N = 0.5 \log(1 - A)^2/A + 0.5 \log 2C_0/K$, where C_N = concentration of nucleophile (mol/l.), C_0 = initial concentration of chlorophyll (mol/l.), and A = area of C=O---Mg absorption peak/maximum area of C=O---Mg peak. The linearity and slope one-half of the plots show that the disaggregation of chlorophyll dimers to form chlorophyll monomer monosolvate comprises an important portion of the over-all reaction. Additional data are given in Table I: A, CH_3OH titration of chlorophyll a in CCl_4; B, tetrahydrofuran (THF) titration of chlorophyll a in CCl_4; C, THF titration of chlorophyll a in *n*-butylcyclohexane; D, THF titration of pyrochlorophyll a in *n*-butylcyclohexane.

and dimer forms must be different, with the extinction coefficient in the dimer being considerably the larger. Figure 3C shows the disaggregation of chlorophyll a oligomers in *n*-butylcyclohexane solution by tetrahydrofuran. The spectrum of the chlorophyll monomer with tetrahydrofuran as nucleophile in *n*-butylcyclohexane is seen to be identical with that observed in CCl_4.

In similar fashion Figures 3D, 3E, and 3F show the disaggregation of chlorophyll b, bacteriochlorophyll, and pyrochlorophyll oligomers in *n*-butylcyclohexane solution by tetrahydrofuran. In each instance the final spectrum obtained by titration is that of the chlorophyll monomer.[23] In each instance the free keto C=O absorption appears near 1700 cm^{-1}, and the C=O---Mg aggregation peaks at 1652 cm^{-1} (chlorophyll a), 1643 cm^{-1} (bacteriochlorophyll), and 1650 cm^{-1} (pyrochlorophyll a) diminish in area. With bacteriochlorophyll a decrease is observed at both 1613 and 1643 cm^{-1}, indicating that two kinds of C=O---Mg interactions are lost. Again, gain and loss in area do not balance, indicating marked differences in extinction coefficients in the various states of aggregation. The spectra of pyrochlorophyll a show some anomalous features. In the dry, aggregated solutions, the ester C=O absorption maximum is at 1737 cm^{-1}, the free keto C=O absorption is at 1695 cm^{-1}, the aggregation C=O---Mg peak is at 1650 cm^{-1}, and the skeletal modes are at 1615 cm^{-1}. The shoulder at 1708 cm^{-1} is distinct and reproducible; it appears in other pyro compounds (which lack the carbomethoxy group), but we have no assignment to suggest for this shoulder. The comparatively large increase in the area of the free keto C=O absorption peak at 1696 cm^{-1}, which results from the addition of tetrahydrofuran, and the relative areas of the free and aggregated C=O peaks in the pyrochlorophyll a oligomers suggest to us that pyrochlorophyll a is more highly aggregated than is chlorophyll a. Elimination of the carbomethoxy group at position 10 presumably has reduced the steric barriers to oligomer formation.

The titration data do not lend themselves to exact calculations of equilibrium constants, primarily because of changes in extinction coefficient with degree of aggregation and, perhaps even more important, the statistical nature of size distribution of aggregates in *n*-butylcyclohexane solution. Nevertheless, it is possible to make some semiquantitative deductions from the titration data about the relative strength of chlorophyll–chlorophyll and chlorophyll–nucleophile interactions. For CCl_4 solutions of chlorophyll a, where the chlorophyll a is originally present mostly as dimer, the titration data are readily fitted to the equilibrium $Chl_2 + 2L \rightleftharpoons 2Chl \cdot L$. For solutions in *n*-butylcyclohexane, the initial state of aggregation cannot yet be specified, and there is no good way to account for the equilibrium $nChl_2 \rightleftharpoons (Chl_2)_n$ that is also involved in the chlorophyll–nucleophile interaction. Consequently, in these solutions, equilibrium constants have been deduced for only that portion of the titration that fits the equilibrium $Chl_2 + 2L \rightleftharpoons Chl \cdot L$. To calculate the equilibrium constants, the spectra were resolved by a Du Pont curve resolver. The decrease in the area of the aggregation peak was the measure used to determine the concentration of chlorophyll dimer. (The band shift and change in band shape for free keto C=O makes additional difficulties in using the free keto C=O absorption peak for this purpose.) Although we used peak areas, peak heights give the same result. The

(23) Addition of more THF beyond this point affects only the skeletal absorption peak at ~1610 cm^{-1}. We have observed that magnesium-containing chlorophyll derivatives have a skeletal vibration absorption maximum that is sensitive to the concentration of nucleophile. This may be related to the formation of disolvates under conditions of high nucleophile concentration.

equilibrium constants were derived by a graphical procedure and are collected in Table I. Figure 4 shows the graphical procedure used to derive the equilibrium constants. No equilibrium constants have been calculated for chlorophyll b and bacteriochlorophyll, since they show a decrease in *two* aggregation peaks on addition of nucleophiles and the structures of the aggregated forms are not yet established.

The value of 45 l. mol^{-1} for the chlorophyll a–methanol equilibrium in CCl_4 found by the ir titration procedure here agrees very well with the value 56 l. mol^{-1} found by an nmr procedure based on ring-current effects of dihydroporphyrin[7] and is roughly of the same order of magnitude as the value of about 200 l. mol^{-1} found by Rappaport.[17] The value $K_1 = 22$ indicates that tetrahydrofuran appears to be a somewhat weaker base than is CH_3OH, but the difference in base strength is not great. Taking the equilibrium constants in Table I at face value, we conclude that the tendency for the various chlorophylls to form aggregates is markedly enhanced in *n*-butylcyclohexene relative to CCl_4, and that pyrochlorophyll a forms much stronger dimers than chlorophyll a does. Although we have not calculated equilibrium constants for chlorophyll b and bacteriochlorophyll interactions, the amount of tetrahydrofuran needed for full disaggregation of these chlorophylls allows the conclusion that chlorophyll b and bacteriochlorophyll are electrophilic to about the same extent as is chlorophyll a.

Addition of tetrahydrofuran to a 10^{-3} *M* solution of pheophytin a in *n*-butylcyclohexane does not cause any change in the ir spectrum in the carbonyl region.

Chlorophyll–Water Interactions. Figure 5A shows the effect of added water on the state of aggregation of chlorophyll a in CCl_4 solution. Because the solubility of water in CCl_4 is limited (0.008 *M* at saturation[24]), a concentrated chlorophyll a solution in CCl_4 (say 0.1 *M* or greater) is only slightly affected by the water. In a dilute chlorophyll a solution, however, such as shown in Figure 5A, where the concentration of the water is double that of the chlorophyll, there is clear evidence for the disaggregation of the chlorophyll by nucleophilic interaction of the water with the magnesium of chlorophyll a. The keto C=O---Mg aggregation peak at 1652 cm^{-1} diminishes, and the free keto C=O peak at 1696 cm^{-1} increases in intensity with added water.

The chlorophyll b–water interaction in CCl_4 solution is strongly concentration dependent (Figures 5B and 5C). Whereas a dilute (3.2×10^{-3} *M*) solution of chlorophyll b (Figure 5B) shows only a slight effect on addition of the water, only a sharpening of the 1667-cm^{-1} band and a tailing to lower wave number of the 1738-, 1703-, and 1665-cm^{-1} absorptions being observed, the effects are quite strong on a 2×10^{-2} *M* solution of chlorophyll b. The free keto C=O absorption peak at 1703 cm^{-1} decreases, the keto C=O---Mg interaction at 1665 cm^{-1} likewise decreases, and the decreases in the intensity of the peak at 1610 cm^{-1} suggest that any aldehyde C=O---Mg interaction has similarly been interrupted by the addition of water. Accompanying the decreases in free keto absorption at 1703 cm^{-1} is an increase in the free aldehyde C=O absorption at ~1680 cm^{-1}, and the appearance of a strong peak at 1645 cm^{-1} which, as we shall show below, can be ascribed to a keto C=O---HO(H)---Mg interaction. With respect to formation of chlorophyll–water micelles in CCl_4, chlorophylls a and b thus differ significantly.

In bacteriochlorophyll–CCl_4 solutions, water appears to disrupt acetyl C=O---Mg but not the keto C=O---Mg interactions (Figure 5D). No increase in the free keto C=O absorption is noted here on addition of water, but the free acetyl C=O contribution included in the peak at 1655 cm^{-1} is enhanced. We can summarize and generalize these observations by saying that for all of the chlorophylls, in any nonpolar solvent, there will be a chlorophyll concentration sufficiently small so that enough of an excess of water can be attained to disaggregate the chlorophyll by nucleophilic interaction.

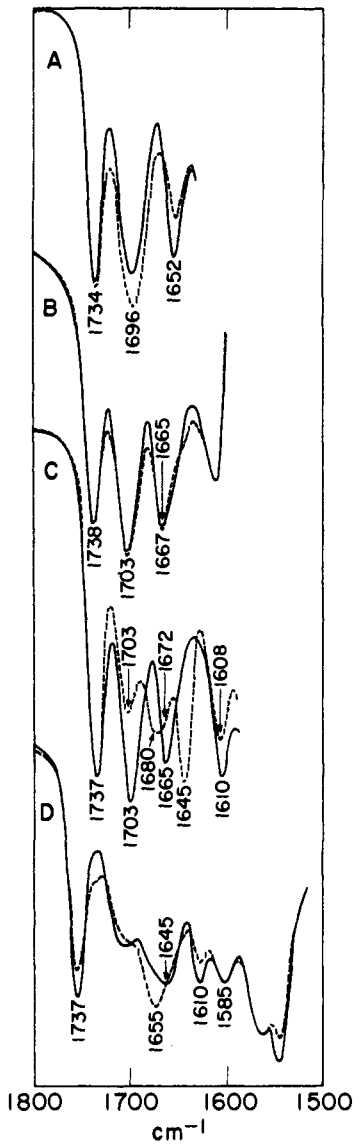


Figure 5. Effect of water on ir spectra (1800–1500-cm^{-1} region) of chlorophylls dissolved in CCl_4: A, 4.0×10^{-3} *M* chlorophyll a (1 mm cell), (——) dry, (– – –) water saturated; B, 3.2×10^{-3} *M* chlorophyll b (1 mm cell), (——) dry, (– – –) water saturated; C, 2.0×10^{-2} *M* chlorophyll b (0.2 mm cell), (——) dry, (– – –) water saturated; D, 3.0×10^{-2} *M* bacteriochlorophyll (0.15 mm cell), (——) dry, (– – –) water saturated.

(24) J. R. Johnson, S. D. Christian, and H. E. Aufsprung, *J. Chem. Soc., A*, 77 (1966).

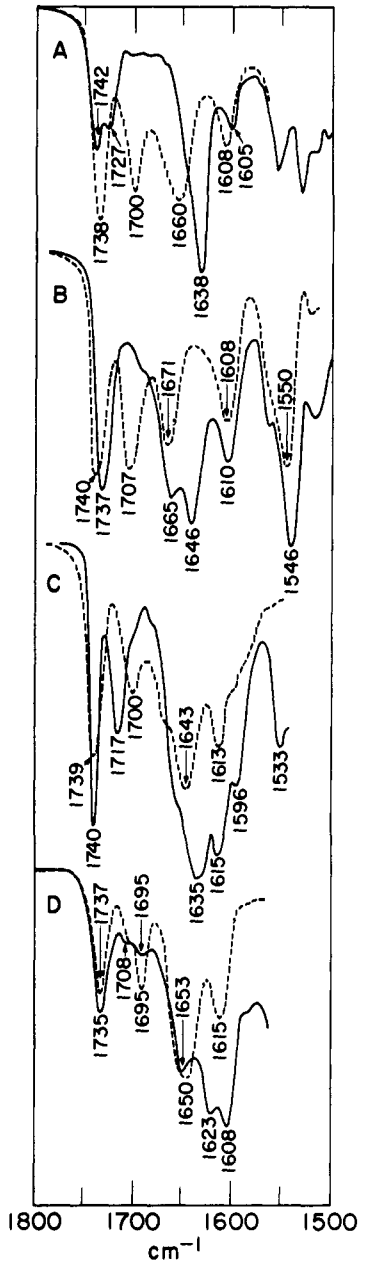


Figure 6. Comparison of ir spectra (1800–1500-cm^{-1} region) of anhydrous chlorophylls and chlorophyll–water micelles in aliphatic hydrocarbon solution. Concentration of the chlorophylls is about 5×10^{-3} *M*. Intensities cannot be compared quantitatively between anhydrous and hydrated chlorophyll solutions: A, (– – –) anhydrous chlorophyll a in NBC, (——) hydrated chlorophyll a (1 chlorophyll:1H_2O) in hexadecane solution; B, (– – –) anhydrous chlorophyll b in NBC, (——) hydrated chlorophyll b (hydrated in cyclohexane with excess water) in NBC solution (compare 5C); C, (– – –) anhydrous bacteriochlorophyll in NBC, (——) hydrated bacteriochlorophyll (hydrated in cyclohexane with excess water) in NBC; D, (– – –) anhydrous pyrochlorophyll a in NBC, (——) hydrated pyrochlorophyll a (hydrated in cyclohexane with excess water) in hexadecane solution.

The less soluble the dry chlorophyll in the particular solvent chosen, the lower this chlorophyll concentration must be. Obviously, the lower the solubility of the water in the nonpolar solvent, the lower the chlorophyll concentration will need to be to detect disaggregation. In nonpolar solvents in which water is reasonably soluble, such as benzene, carbon tetrachloride, or chloroform, it is easy to arrange conditions so that the concentration of the water is very much larger than the chlorophyll. In such circumstances, the chlorophyll aggregates will be partially or completely converted to chlorophyll monomer hydrates.

The effects of solvent on the chlorophyll–water interaction can be seen in Figure 6. In these experiments, the ir spectra of dry and hydrated chlorophylls are compared in aliphatic hydrocarbon solvents. The ir spectra in cyclohexane, *n*-butylcyclohexane, dodecane, and hexadecane are very similar.[25] It is obvious from the spectra of Figure 6 that chlorophyll–water interactions in these solvents involve much more than the nucleophilic disaggregation of chlorophyll–chlorophyll oligomers. A dry chlorophyll a solution in hexadecane shows (Figure 6A) a rather typical oligomer spectrum. However, when water is present in 1:1 ratio to chlorophyll, an intense peak appears at 1638 cm^{-1}. The free keto C=O absorption maximum near 1700 cm^{-1} disappears, and the ester peak is split, with one of the components of the ester C=O shifted to 1727 cm^{-1}, the other retreating to 1742 cm^{-1}. We do not consider that the presence of water causes the keto C=O---Mg absorption maximum to move from 1652 to 1638 cm^{-1}. Rather, we believe that the keto C=O---Mg interaction is essentially completely disrupted, and a new interaction resulting in absorption at 1638 cm^{-1} takes its place. This new absorption we assign to a keto carbonyl–water interaction, in which the water is coordinated to magnesium: C=O---HO(H)---Mg. Repetition of this hydrogen-bonding process will result in the formation of a large aggregate. The ir spectrum in the OH stretch region is highly consistent with this assignment. The interpretation given here of the 1638-cm^{-1} peak implies that the chlorophyll–water species giving rise to the ir spectrum are basically different from those that exist in CCl_4, benzene, or $CHCl_3$ solutions of comparable chlorophyll–water composition. The visible absorption spectra, X-ray, and ultracentrifugation observations likewise fully support this conclusion.[19] We emphasize that the stoichiometry of the interaction has been verified by direct analysis for the chlorophyll–water ratio.

The spectra of water adducts of chlorophyll b, bacteriochlorophyll, and pyrochlorophyll a can be interpreted along the same lines. The spectrum of hydrated chlorophyll b in *n*-butylcyclohexane (Figure 6B) likewise shows marked changes relative to an anhydrous solution. The strong free keto C=O peak at 1707 cm^{-1} seen in dry solutions completely disappears. The keto C=O---Mg aggregation peak at 1670 cm^{-1} (which contains a contribution from free aldehyde C=O) is replaced by a very strong absorption at 1646 cm^{-1}, again assigned to a keto C=O---HO(H)---Mg interaction. The free aldehyde C=O peak is distinctly visible at 1665 cm^{-1}, and its apparent increase in intensity suggests that the formation of the chlorophyll–water species in this solvent is accompanied by the disruption of some previously existent aldehyde C=O---Mg interactions. The shift in position from ~1680 to 1665 cm^{-1} compared to the chlorophyll b–water (1:1) aggregate in CCl_4 suggests that water may also be hydrogen bonded to the aldehyde C=O, as the hydration in *n*-butylcyclohexane was

(25) Nevertheless, it should not be inferred that all aliphatic or cycloaliphatic solvents are entirely equivalent. For example, hexadecane and Nujol, which contains large amounts of naphthenes, are similar, but by no means identical solvents.

carried out with excess water. Although there is a slight shift in the position of the ester C=O peaks (from 1740 to 1737 cm^{-1}), no splitting is observed in the chlorophyll b–H_2O adduct. This argues for a basically different orientation of dihydroporphyrin ring and water for the b adduct as compared to that of chlorophyll a. Bacteriochlorophyll–water interactions (Figure 6C) are also interpretable on this basis. The formation of a bacteriochlorophyll–water aggregate results in the disappearance of the free keto C=O absorption peak seen in bacteriochlorophyll trimers or oligomers at 1700 cm^{-1}, again indicating that no free keto C=O exists in the bacteriochlorophyll–water adduct. The peak at 1643 cm^{-1}, assigned to keto C=O---Mg absorption, and containing a contribution from free acetyl C=O, now instead appears as a considerably strengthened absorption maximum at 1635 cm^{-1}, which we again assign to a keto C=O---HO(H)---Mg interaction. Although difficult to judge because of the breadth of the 1635-cm^{-1} peak, the skeletal absorptions at 1613 cm^{-1} (that also contains a coordinated acetyl C=O---Mg contribution) appear somewhat weaker; from this we conclude that formation of the bacteriochlorophyll–water adduct probably occurs with disruption of acetyl C=O---Mg interactions, and that the acetyl group has only a minor part in the formation of the adducts. The ester C=O peak at 1739 cm^{-1} in dry bacteriochlorophyll is split in the bacteriochlorophyll–water adduct, now appearing at 1740 and 1717 cm^{-1}; this circumstance we take to indicate that the structure of the bacteriochlorophyll–water aggregate resembles that of chlorophyll a–water more closely than that of chlorophyll b–H_2O adducts.

The interaction of pyrochlorophyll a with water requires special mention. Although pyrochlorophyll a differs from chlorophyll a only in the absence of the carbomethoxy group at position C-10 where it is replaced by H, the spectral changes occurring on formation of a pyrochlorophyll–water adduct in aliphatic hydrocarbon solvents (Figure 6D) appear very different from those observed with chlorophyll a. The free keto C=O absorption at 1695 cm^{-1}, present in dry pyrochlorophyll a oligomers, is almost completely suppressed, but new peaks appear at 1653 and 1623 cm^{-1}, while the maximum at 1650 cm^{-1} likewise disappears. We believe these spectral changes are best interpreted along the lines that formation of a pyrochlorophyll a–water adduct in *n*-butylcyclohexane results in the disappearance of most of the free keto C=O absorption, but that, unlike the other chlorophylls, coordinated keto C=O---Mg (1653 cm^{-1}) interactions persist in the adduct, along with coordinated keto C=O---HO(H)---Mg (1623 cm^{-1}). This conclusion is supported by the absorption spectrum in the visible, where the red absorption maximum for anhydrous pyrochlorophyll a cannot be shifted completely by addition of water, as is the case with chlorophylls a and b and bacteriochlorophyll. The skeletal vibrations in the pyrochlorophyll a–H_2O adduct are displaced from 1615 to 1608 cm^{-1} in the water adduct. The ester C=O peak is unchanged. All of the changes are consistent with the conclusion that the pyrochlorophyll a–water interaction product is very different from that of chlorophyll a–water adducts. It also suggests that the carbomethoxy group in chlorophyll a is an important component in the structural integrity of the chlorophyll a–water aggregate.

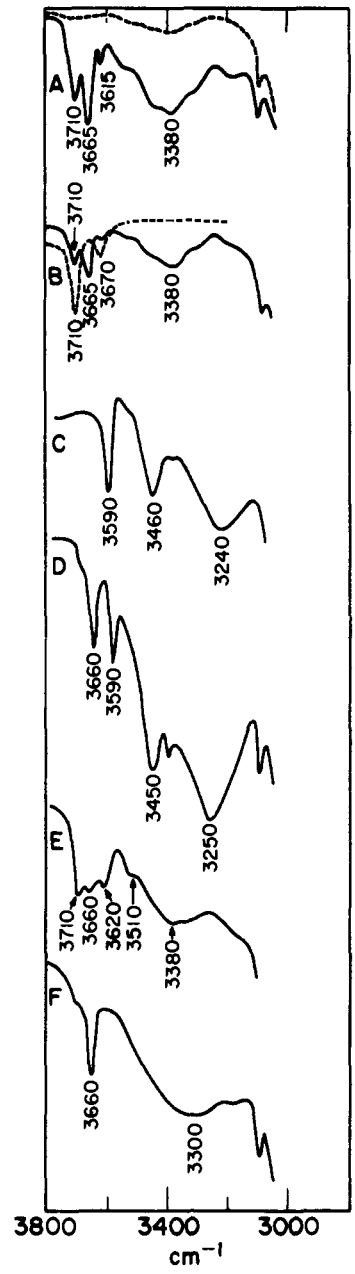


Figure 7. Ir spectra in the 3800–3000-cm^{-1} region of hydrated chlorophylls in CCl_4 and hexadecane solutions: A, (——) chlorophyll a–H_2O (1:1) in CCl_4, 6×10^{-3} *M* (1.5 mm cell), (– – –) anhydrous chlorophyll a in dry CCl_4; B, (——) anhydrous chlorophyll a (8×10^{-3} *M*) in water-saturated CCl_4 (8×10^{-3} *M*) (1 mm cell), (– – –) water-saturated CCl_4 (1 mm cell); C, mull of chlorophyll a–H_2O (1:1) in hexadecane (about 10^{-1} *M*); D, highly concentrated chlorophyll a–H_2O (1:1) in CCl_4 (1 mm cell); E, chlorophyll b–H_2O (2:1–3:2) in CCl_4 ($1.6\ M\ 10^{-3}$, 10 mm cell); F, pyrochlorophyll (hydrated in cyclohexane with excess water) in CCl_4 (2×10^{-3} *M*, 10 mm cell).

Ir Spectra in the OH Stretch Region. Because of the major changes in the state of water postulated to occur on formation of chlorophyll–water adducts, it becomes of interest to examine the OH stretch region of the ir. We have therefore studied the OH region in CCl_4 and aliphatic hydrocarbon solutions of the chlorophylls, and have repeated and extended the observations of Rappaport[17] and Sidorov and Terenin[26] in this region. To

(26) A. H. Sidorov and A. H. Terenin, *Opt. Spectry. USSR*, **8**, 254 (1960).

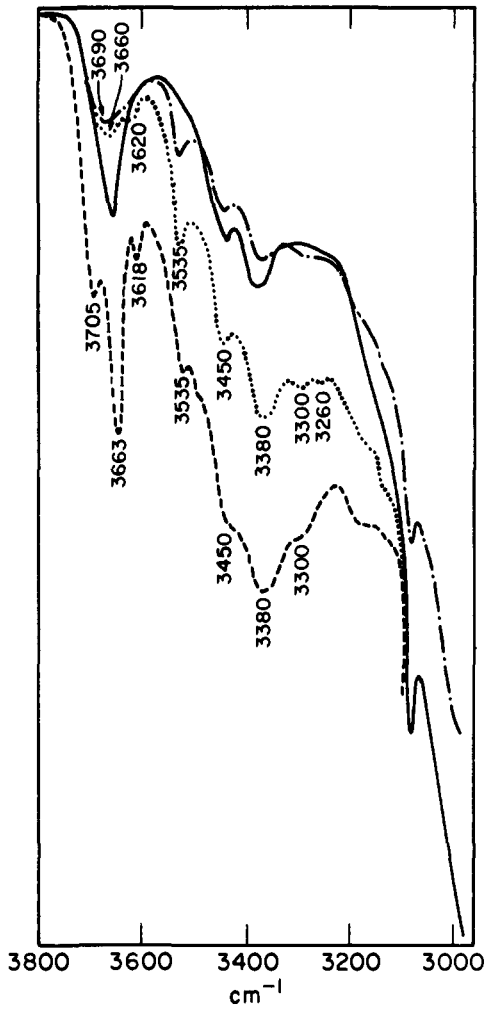


Figure 8. Exchangeable hydrogen from ir spectra in the 3800–3000-cm^{-1} region of concentrated chlorophyll a solution (10 mm cell): A, (– – –) chlorophyll a–H_2O (1:1), 10^{-2} *M* in CCl_4, 10 mm cell; B, (···) same as A, but dried by codistillation with CCl_4; C, (–·–·–) chlorophyll a, dried by codistillation with CCl_4 and heating at 60° for 1 hr (10^{-2} *M* in CCl_4, 10 mm cell); D, (——) same as C, shaken with D_2O.

observe the water region, concentrated solutions and long light paths are required. Assignments are based on those of Mecke, *et al.*,[27] and Barrow, *et al.*[28] Chlorophyll a monohydrate dissolved in dry CCl_4 (Figure 7A) shows the antisymmetric OH stretch fundamental vibration ν_3 at 3710 cm^{-1} and the symmetric stretch ν_1 at 3615 cm^{-1} characteristic of monomeric water in CCl_4. The presence of these peaks indicates that free water has been produced according to equilibria 3 and 6 (see Discussion) from chlorophyll monomer or dimer hydrates at the same time the carbonyl ir spectra clearly indicate the presence of chlorophyll dimers. Between the free water peaks is an absorption maximum at 3665 cm^{-1}, which appears only when both chlorophyll and water are present. In agreement with Rappaport,[17] we attribute this peak to chlorophyll dimer or chlorophyll monomer hydrate(s), and assign it to an OH stretch mode in water coordinated to magnesium: Mg---O(H)H. When the CCl_4 is removed from the solution by pumping under high vacuum, and the residual chlorophyll redissolved in dry CCl_4, a spectrum is obtained which shows very little water absorption (Figure 7A). In this approximately 10^{-1} *M* dry chlorophyll a solution in CCl_4 there is present on a conservative estimate less than 10^{-4} *M* water. Figure 7B shows the absorption spectrum of a saturated (8×10^{-3} *M*) solution of water in CCl_4, together with the spectrum of an 8×10^{-3} *M* chlorophyll a solution in water-saturated CCl_4. Again we see the symmetric and antisymmetric OH stretching modes of H_2O at 3710 and 3610 cm^{-1} in the water-saturated CCl_4. Addition of dry chlorophyll a causes these two maxima to diminish, indicative of a decrease in the concentration of free water, and the peak at 3665 cm^{-1}, which we have assigned to Mg---O(H)H, appears. Poorly defined shoulders appear at the 3380-cm^{-1} absorption maximum, which we assign to Mg---O(H---OH_2)$_2$. The shoulders appear not to involve OH, as exchange with D_2O does not affect them (Figures 8A and 8D). This typical chlorophyll–water spectrum can be taken to indicate the presence of a small amount of unbound water, and the chlorophyll species $Chl_2 \cdot H_2O$ and $Chl \cdot H_2O$ to be present in the CCl_4 solution.

In hexadecane solution we find the situation quite different (Figure 7C). No free water absorption peaks can be seen. The ir spectrum in the carbonyl region of this solution shows strong ester splitting, no free keto C=O, and a very strong absorption peak at 1638 cm^{-1}. A compatible assignment of the water spectrum then is as follows: 3590 cm^{-1}, the free OH stretch in water coordinated to magnesium and hydrogen bonded to a keto group, Mg---O(H)H---O=C keto; the peak at 3460 cm^{-1}, the OH stretch in water coordinated to magnesium and hydrogen bonded to the carbomethoxy C=O, ester $\overline{C{=}O{-}{-}{-}HO(Mg)H{-}{-}{-}O{=}C}$ keto; and the absorption at 3240 cm^{-1} to the hydrogen-bonded keto carbonyl, ester $\overline{C{=}O{-}{-}{-}HO(Mg)H{-}{-}{-}O{=}C}$ keto. These three peaks have also been observed but not assigned by Holt and Jacobs[29] and by Sherman and Wang.[16] The absorption at 3240 cm^{-1} correlates with the absorption at 1638 cm^{-1}. These peaks may be taken as diagnostic of the presence of cholorophyll–water aggregates in which C=O---Mg interactions characteristically present in dimers or oligomers are absent, and in which water is coordinated to the magnesium atom of one chlorophyll molecule and simultaneously hydrogen bonded to the carbonyl groups of the carbomethoxy ester function and the keto carbonyl at position 9 of another chlorophyll molecule. The presence of one of these ir peaks implies the presence of the other. Such solutions are yellow green, with an absorption maximum in the visible at about 740–745 nm. Other evidence indicates that in chlorophyll a solutions showing the 3240-cm^{-1}, 1638-cm^{-1}, and 740-nm absorption maxima, chlorophyll–water adducts of colloidal dimensions are present.[18,19]

The effect of water in chlorophyll a solutions in CCl_4 10^{-3} *M* or less in concentration, as shown in Figure 5A, is partial disaggregation of chlorophyll oligomers to form $Chl_2 \cdot H_2O$. That equilibria are involved can be deduced from Figure 7D, which shows the spectrum of a very concentrated hydrated chlorophyll a solution in CCl_4. A mixture of species is readily discerned to be

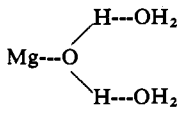
(27) E. Greinacher, W. Lüttke, and R. Mecke, *Z. Elektrochem.*, **59**, 23 (1955).

(28) S. C. Mohr, W. D. Wilk, and G. M. Barrow, *J. Am. Chem. Soc.*, **87**, 3048 (1965).

(29) A. S. Holt and E. E. Jacobs, *Plant Physiol.*, **30**, 553 (1955).

present. No bands originating from free water are to be seen. The band at 3660 cm^{-1} can be assigned to the OH stretch in water coordinated to magnesium (in $Chl_2 \cdot H_2O$ or $Chl \cdot H_2O$): Mg---O(H)H. The other bands are those assigned to magnesium-coordinated water that is simultaneously hydrogen bonded to two carbonyl functions in another chlorophyll molecule. As expected, this solution is yellow green, not blue as is the oligomer, and it also shows an absorption peak at 1638 cm^{-1}. Thus, even in CCl_4, the chlorophyll–water equilibrium can be displaced under forcing conditions in the direction of chlorophyll–water micelle formation.

Figure 7E shows the water region of a hydrated chlorophyll b solution in CCl_4. The vibrations attributable to free water are responsible for the peaks at 3710 (ν_3) and 3620 (ν_1) cm^{-1}. The peak at 3660 cm^{-1} is weak and the broad absorption at 3380 cm^{-1} is assigned to



Hydrated pyrochlorophyll a in CCl_4, judged from the carbonyl region spectrum, which shows a weak free keto C=O and a strong 1643-cm^{-1} absorption, forms pyrochlorophyll a–water micelles. In the water region (Figure 7F), only absorption peaks at 3660 and 3300 cm^{-1} are seen, consistent with the absence of the carbomethoxy C=O---HO(H)---Mg stretching mode.

Because the concentrated chlorophyll solutions in CCl_4 that are used to examine the water region show absorption maxima near 3535, 3450, 3380, and 3300 cm^{-1} even when the chlorophyll and the solvent were dried (Figure 8C), exchange experiments with D_2O have been carried out to see whether these absorptions involve OH vibrational modes. It is clear from Figure 8D that most of the absorption peaks in the region 3440–3200 cm^{-1} do not involve hydrogenic modes; they are probably C=O overtones. The peak at 3535 cm^{-1}, which is rapidly lost by exchange with D_2O, is an exception. We believe it arises from the enol form of chlorophyll a, and this peak is discussed in more detail below. The water bands and the magnesium-coordinated OH that appear arise by absorption and exchange of ordinary H_2O from the atmosphere, as no precautions were taken to minimize adventitious water in this experiment.

Enol Form of Chlorophyll a. Nmr studies of hydrogen exchange in chlorophyll are best explained on the basis that the predominant form of the chlorophylls in solution is the keto form.[30] Nevertheless, the ring V system of chlorophyll is a β-keto ester, and the existence of 10-epi-diastereoisomeric forms of chlorophyll[31] can best be accounted for by a mechanism involving the enolization of the hydrogen at position C-10. Sidorov and Terenin[26] and Karyakin and Chibisov[32] attributed absorption at 3525 cm^{-1} to the enol form of chlorophyll a, in dry benzene or chloroform solutions. We, too, were able to show that the absorption maximum at 3530 cm^{-1},

(30) R. C. Dougherty, H. H. Strain, and J. J. Katz, *J. Am. Chem. Soc.*, **87**, 104 (1965).
(31) J. J. Katz, G. D. Norman, W. A. Svec, and H. H. Strain, *ibid.*, **90**, 6841 (1968).
(32) A. V. Karyakin and A. K. Chibisov, *Opt. Spectry. USSR*, **13**, 209 (1962).

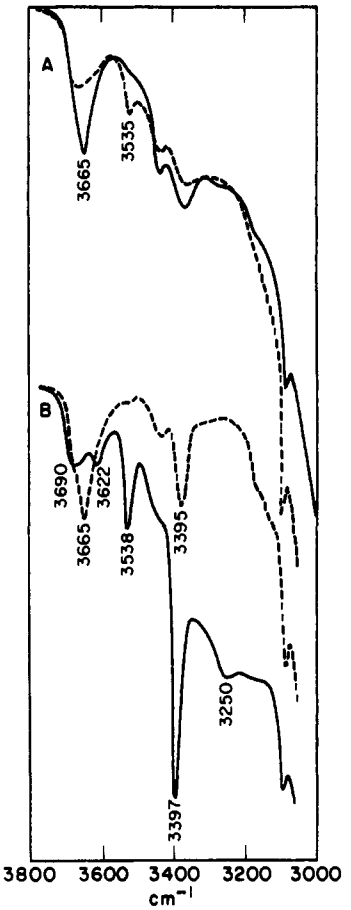


Figure 9. Ir spectra of enol form of chlorophyll a. Changes in 3800–3000-cm^{-1} region of chlorophyll a and pheophytin a upon D_2O exchange: A, (- - -) anhydrous chlorophyll a in CCl_4 (10^{-2} *M*, 10 mm cell), (——) D_2O exchange (15 min); B,(——) pheophytin a in CCl_4 (7×10^{-3} *M*, 10 mm cell), (- - -) D_2O exchange (40 min).

which can be observed in very concentrated dry solutions of chlorophyll a, is exchangeable with D_2O. In Figure 9A the small absorption peak at 3535 cm^{-1} is seen to disappear very rapidly on addition of D_2O, establishing its identity as an O–H group. This absorption peak is also seen in concentrated pheophytin solutions in CCl_4 (Figure 9B). Here, the absorption peaks at 3538 and 3397 cm^{-1} are both exchangeable with D_2O, the peak at 3538 cm^{-1} rapidly and the one at 3397 cm^{-1} slowly; the peak at 3665 cm^{-1} arises from OH introduced by exchange with D_2O during the experiments. The amount of enolic chlorophyll a in CCl_4 solution must be very small, probably considerably less than 1% as judged by the fact that very concentrated solutions are required to see the 3538-cm^{-1} peak at all.

Effect of Hydrogen-Bonding Agents on the Carbonyl Ir Spectra. Hydroxylic solvents are known to cause changes in the ir spectra of chlorophyll dissolved in nonpolar solvents.[8,16] Some of the changes are in addition to those expected from an electron donor only and must arise from the ability of the ROH molecule to participate in hydrogen bonding. Figure 10A shows the spectrum of monomeric chlorophyll a in an *n*-butylcyclohexane-2% tetrahydrofuran mixture; only one ester C=O absorption occurs, free keto C=O at 1705 cm^{-1} is very

strong, and no aggregated C=O---Mg absorption is apparent. Upon addition of about a 100-fold stoichiometric excess of methanol, the ester absorption peak splits, now appearing at 1742 and 1715 cm^{-1}. The shoulder at 1715 cm^{-1} we assign to hydrogen-bonded ester C=O---$HOCH_3$. The free keto C=O diminishes in intensity, and the shoulder at 1680 cm^{-1} can in turn be assigned to keto C=O---$HOCH_3$. An analogous interpretation can be made of the effects of CH_3OH on the monomeric chlorophyll b spectrum (Figure 10B). The ester peaks are split, and the shoulder at 1720 cm^{-1} is attributed to ester C=O---$HOCH_3$; the free keto C=O absorption decreases, and the slight shoulder and broadening at 1685 cm^{-1} is assigned to keto C=O---$HOCH_3$. The free aldehyde C=O at 1672 cm^{-1} appears largely unaffected by the addition of alcohol, but hydrogen bonding of the aldehyde C=O may be masked by shift of the 1710-cm^{-1} peak to 1685 cm^{-1}. In any event, we construe the inflection at 1655 cm^{-1} to indicate the presence of some aldehyde C=O---$HOCH_3$ interactions in the hydrogen-bonded species.

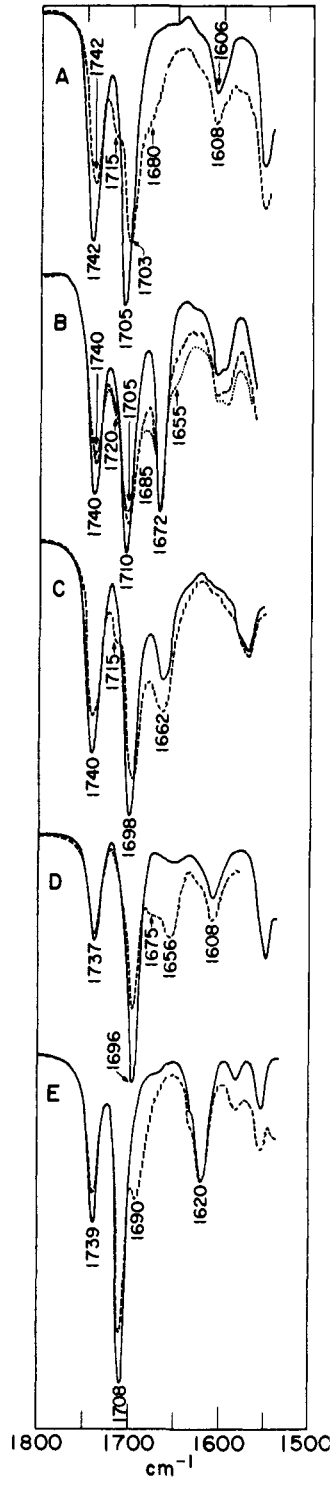


Figure 10. Effect on ir spectra of hydrogen bonding by CH_3OH to carbonyl functions in chlorophylls, 1800–1500-cm^{-1} region: A, (——) chlorophyll a in NBC–2% THF (1.0×10^{-3} *M*, 1.77 mm cell), (– – –) 1% CH_3OH added; B, (——) chlorophyll b in NBC–2% THF (2.2×10^{-3} *M*, 1.77 mm cell), (– – –) 0.6% CH_3OH added, (· · ·) 1.2% CH_3OH added; C, (——) bacteriochlorophyll in NBC–2% THF (1.7×10^{-3} *M*, 1.77 mm cell), (– – –) 0.4% CH_3OH added; D, (——) pyrochlorophyll a in NBC–2% THF (2.0×10^{-3} *M*, 1.77 mm cell), (– – –) 0.3% CH_3OH added; E, (——) pyropheophytin a in NBC–2% THF (2.0×10^{-3} *M*, 1.77 mm cell), (– – –) 1% CH_3OH added.

In the bacteriochlorophyll spectra so far discussed, it appears that the acetyl C=O group is involved in the aggregation to only a minor extent. At least, extensive participation by the acetyl group need not be invoked to account for the data. A similar situation is found to exist with respect to the interaction of bacteriochlorophyll with hydroxylic solvents (Figure 10C). Addition of methanol to monomeric bacteriochlorophyll causes the ester absorption peak to split, as in other chlorophylls, and the shoulder at 1715 cm^{-1} can be associated with an ester C=O---$HOCH_3$ interaction. The free keto C=O at 1698 cm^{-1} decreases and in part shifts to 1662 cm^{-1}, an absorption we assign to keto C=O---$HOCH_3$. Hydrogen bonding to acetyl C=O is much less evident in the spectra than is the case for keto C=O, and it is possible to account for the spectra on the basis that acetyl C=O is weakly or not at all hydrogen bonded to CH_3OH in these solutions.

The addition of methanol to monomeric pyrochlorophyll a (Figure 10D) reveals some unusual features. No splitting of the ester C=O absorption results. This strongly suggests that the ester splitting commonly observed in the other chlorophylls results from preferential hydrogen bonding to the carbomethoxy C=O group. The free keto C=O absorption at 1696 cm^{-1} diminishes; a shoulder or inflection appears at 1675 cm^{-1} and a new peak appears at 1656 cm^{-1}. The former we can assign to keto C=O---$HOCH_3$; an alternative, but in our view less likely possible, is that the 1675-cm^{-1} inflection results from an ester C=O---$HOCH_3$ hydrogen-bonding interaction. The 1656-cm^{-1} peak can arise from hydrogen bonding to methanol coordinated to magnesium: keto C=O---HO(Mg)CH_3. Methanol coordinated to Mg^{2+} is known to be much more acidic than bulk methanol,[33] and should therefore form stronger hydrogen bonds. That magnesium may be involved in the appearance of the peak at 1656 cm^{-1} is supported by observations on pyropheophytin a (Figure 9E). Some decrease in ester absorption is noted here; presumably the ester C=O---$HOCH_3$ absorption is buried under the free keto C=O peak at 1708 cm^{-1}. The peak at 1690 cm^{-1} can

(33) S. Nakamura and S. Meiboom, *J. Am. Chem. Soc.*, **89**, 1765 (1967).

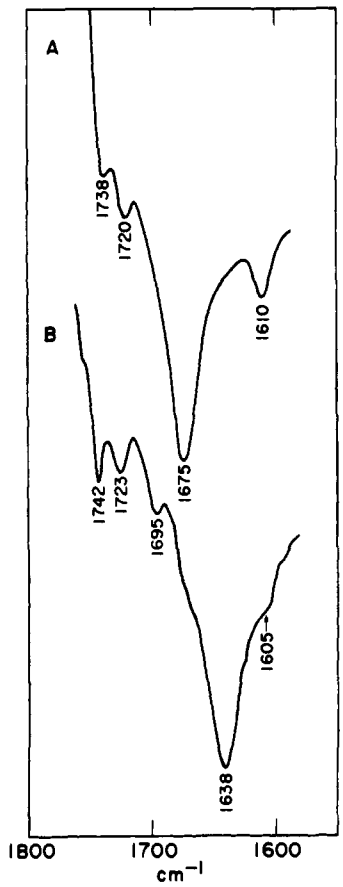


Figure 11. Effect of extensive hydrogen bonding on ir spectra in 1800–1600-cm^{-1} region of chlorophyll a: A, anhydrous chlorophyll a in isoamyl alcohol (10^{-2} *M*, 0.2 mm cell); B, chlorophyll a–H_2O sonicated with excess water in hexadecane (about 10^{-2} *M*, 0.2 mm cell).

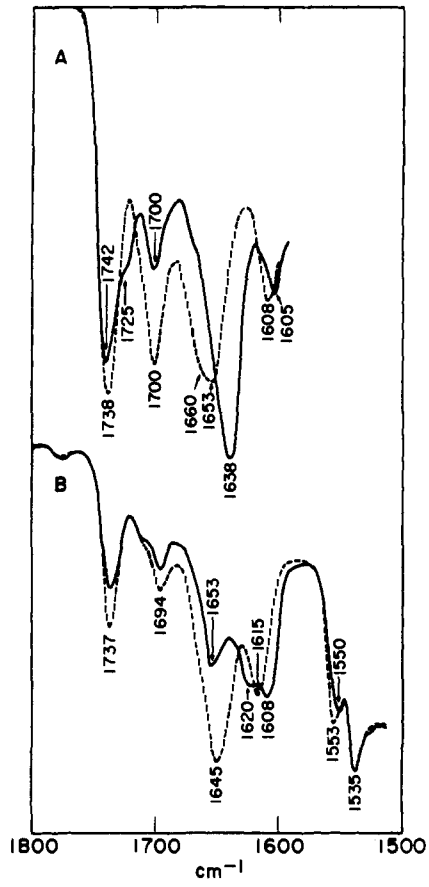


Figure 12. Effect of temperature on chlorophyll–water micelles in aliphatic hydrocarbon solvents, 1800–1600-cm^{-1} region: A, (——) chlorophyll a–H_2O (2.2×10^{-2} *M*, 0.2 mm cell) in heptylcyclohexane solution, (– – –) heated at 65° for 2 min; B, (——) hydrated pyrochlorophyll a (0.8×10^{-2} *M*, 0.2 mm cell) in NBC, (– – –) heated at 65° for 2 min.

then be assigned to keto C=O---$HOCH_3$. No peak is seen at 1656 cm^{-1}, consistent with the supposition that Mg---(H)OCH_3 is involved in the appearance of this absorption peak in pyrochlorophyll a.

Finally, the spectrum of fully hydrogen-bonded chlorophyll a in solution is shown in Figure 11A. The spectrum is quite featureless. The very broad peak centered at 1675 cm^{-1} must include the ester C=O hydrogen bonds as well as the keto C=O---HOR contribution. Comparison with maximally hydrated chlorophyll is instructive (Figure 11B). When excess water, sufficient to form a separate phase, is added to chlorophyll–water (1:1) micelles in hexadecane, a precipitate forms. The ir spectrum of such a precipitate is shown in Figure 11B. The ester C=O absorptions are split and appear at 1742 and 1723 cm^{-1}; very little free keto C=O (1695 cm^{-1}) is evident, and all of these peaks, including the skeletal modes at 1605 cm^{-1} are dwarfed by the enormous peak at 1638 cm^{-1}. The spectra in Figure 11 thus confirm the important differences between the interactions of chlorophyll a with ROH and with HOH. It is only the latter that can form the species that give rise to the absorption maximum at 1638 cm^{-1}.

Temperature Effects. If chlorophyll–water adducts in aliphatic hydrocarbon solvents do have an organized structure, then heating should disrupt the chlorophyll–water aggregates or micelles. Such is the case. In Figure 12A, the results of heating a chlorophyll–water (1:1) solution in heptylcyclohexane for 2 min at 60° are seen to be the disappearance of the 1638-cm^{-1} peak (characteristic of the ester C=O---HO(Mg)H--O=C keto interaction) and reversion to the dry oligomer spectrum with a keto C=O---Mg aggregation peak at 1653 cm^{-1}. Reconstitution of the chlorophyll a–water micelle on cooling is very slow. In the case of pyrochlorophyll a–water micelles, heating at 65° for 2 min also disrupts the micelles (1653 and 1620 cm^{-1}) to oligomers (1645 cm^{-1}) (Figure 12B). However, these micelles are fully reconstituted in 1 hr at room temperature. This observation strengthens our previous conclusions that the chlorophyll a–water and pyrochlorophyll a–water micelles have basically different structures.

Discussion

The ir experimental data, summarized in Tables II, III, and IV, provide a basis for conclusions about the nature of the chlorophyll species that occur in nonpolar solvents in the presence and absence of water. Various monomeric and aggregated chlorophyll species can be shown to exist, depending on the nonpolar solvent, chlorophyll concentration, and the particular chlorophyll involved. The relationships of the various species to each other are best systematized by a set of equilibria.

Table II. Absorption Bands (1800–1600 cm^{-1}) in Chlorophyll Dimer and Oligomer (cm^{-1})

Compound	Solvent	Ester C=O	Keto C=O	Coordinated keto[a] C=O---Mg	Skeletal (C=C)/(C=N)
Chlorophyll a	Carbon tetrachloride	1735	1695	1652	1608
Chlorophyll a	*n*-Butylcyclohexane	1740	1703	1662,[e] 1652	1610
Chlorophyll b	Carbon tetrachloride	1738	1703	1665[b]	1608[c]
Chlorophyll b	*n*-Butylcyclohexane	1740	1707	1672[b]	1608[c]
Bacteriochlorophyll	Carbon tetrachloride	1736	1685	1645[d]	1610
Bacteriochlorophyll	*n*-Butylcyclohexane	1739	1700	1645[d]	1613
Pyrochlorophyll a	*n*-Butylcyclohexane	1737	1695	1650	1615

[a] Elsewhere this has been referred to as "aggregated ketone carbonyl." [b] Contains a contribution from free aldehyde C=O. [c] Contains a contribution from magnesium-coordinated aldehyde C=O: aldehyde C=O---Mg. [d] Contains a contribution from free acetyl C=O at 1655 and 1663 cm^{-1}, respectively. [e] C=O---Mg interaction in oligomers.

Table III. Absorption Bands (1800–1600 cm^{-1}) in Chlorophyll–Water Micelles[a] (cm^{-1})

Compound	Propionic ester C=O	H-bonded carbomethoxy C=O---HO(Mg)H	H-bonded keto C=O---HO(Mg)H	Skeletal (C=C)/(C=N)
Chlorophyll a	1742	1727	1638	1605
Chlorophyll b	1737	...	1645[b]	1610
Bacteriochlorophyll	1740	1717	1635[c]	1615
Pyrochlorophyll a[d]	1735	...	1623	1608

[a] In *n*-butylcyclohexane or hexadecane solution. [b] Aldehyde C=O appears at 1665 cm^{-1}. [c] Contains a contribution from free acetyl C=O. [d] A peak at 1653 cm^{-1} is assigned to magnesium-coordinated keto: C=O---Mg.

Table IV. OH Absorption Bands (3800–3200 cm^{-1}) in Chlorophyll–Water Adducts (cm^{-1})

Species	Solvent	Free HOH	Mg---OH_2	Free OH in singly H-bonded H---O=C, Mg---O, H	Doubly H-bonded H---O=C, Mg---O, H---O=C: Ester	Doubly H-bonded: Keto
(Chl a·H_2O)$_n$[a]	CCl_4	3710 ν_3 3620 ν_1	3665 3380[b]		...	...
(Chl a·H_2O)$_n$[a]	$C_{16}H_{34}$	...	...	3590	3460	3240
(Chl b·H_2O)$_n$[c]	CCl_4	3710 ν_3 3620 ν_1	3660 3380[b]		...	...

[a] Starting with solid chlorophyll monohydrate, Chl·H_2O (1:1). [b] Assigned to Mg---O(H---OH_2)$_2$. [c] Composition range 3Chl b: $2H_2O$ to 3Chl b:$1H_2O$.

The equilibria are subject to the law of mass action, and the concentration of the various species is determined, as is the case for any equilibrium, by the equilibrium constants, concentration of the components, and the temperature.

Equilibria. In nonpolar solvents we can write equilibria 1–9 for the chlorophyll–water interaction.

$$2\text{Chl} \overset{K_1}{\rightleftharpoons} \text{Chl}_2 \quad (1)$$

$$n(\text{Chl}_2) \overset{K_2}{\rightleftharpoons} (\text{Chl}_2)_n \quad (2)$$

$$\text{Chl}_2 + \text{H}_2\text{O} \overset{K_3}{\rightleftharpoons} \text{Chl}_2\cdot\text{H}_2\text{O} \quad (3)$$

$$\text{Chl}_2\cdot\text{H}_2\text{O} + \text{H}_2\text{O} \overset{K_4}{\rightleftharpoons} \text{Chl}_2\cdot(\text{H}_2\text{O})_2 \quad (4)$$

$$\text{Chl}_2\cdot(\text{H}_2\text{O})_2 \overset{K_5}{\rightleftharpoons} 2\text{Chl}\cdot\text{H}_2\text{O} \quad (5)$$

$$\text{Chl}_2 + 2\text{H}_2\text{O} \overset{K_6 = K_3K_4K_5}{\rightleftharpoons} 2\text{Chl}\cdot\text{H}_2\text{O} \quad (6)$$

$$\text{Chl}\cdot\text{H}_2\text{O} + \text{H}_2\text{O} \overset{K_7}{\rightleftharpoons} \text{Chl}\cdot(\text{H}_2\text{O})_2 \quad (7)$$

$$n(\text{Chl}\cdot\text{H}_2\text{O}) \overset{K_8}{\rightleftharpoons} (\text{Chl}\cdot\text{H}_2\text{O})_n \quad (8)$$

$$(\text{Chl}\cdot\text{H}_2\text{O})_n + m\text{H}_2\text{O} \overset{K_9}{\rightleftharpoons} (\text{Chl}\cdot\text{H}_2\text{O})_n\cdot(\text{H}_2\text{O})_m \quad (9)$$

Because most of the information now available is on chlorophyll a, the equilibria have been written with this chlorophyll in mind. In the following, the equilibria are discussed in detail only for chlorophyll a.

Equilibrium 1 is displaced very far to the right in any nonpolar solvent at room temperature. The ir and nmr data indicate that the equilibrium constant K_1 for this reaction must be at least 10^5 or even 10^6.[34] The co-

(34) Sauer, Smith, and Schultz[35] reported a value of (1.0 ± 0.4) ×

ordination interaction force is strong, and monomeric chlorophyll a must be present in vanishingly small concentrations in any dry and pure nonpolar solvent.

Reaction 2 describes a further aggregation of dimers that can occur under some conditions. In CCl_4, this equilibrium is displaced strongly to the left, a conclusion based primarily on molecular weight and ir measurements,[2] even in chlorophyll solutions as concentrated as 0.1 M.[22] In aliphatic or cycloaliphatic hydrocarbon solvents, at concentrations greater than 10^{-3} M, a significant concentration of aggregates higher than dimer are judged to form. This conclusion derives from the appearance of the coordinated keto C=O---Mg peak at 1660 cm^{-1} in *n*-butylcyclohexane or dodecane. In a 10^{-3} M solution in *n*-butylcyclohexane, the dimer peak at 1652 cm^{-1} shows only a slight shoulder to shorter wavelengths. The coordination interaction holding the dimers together must also involve C=O---Mg. It is likely that there is a small but measurable difference in energy between the interaction that forms dimers and the interaction that forms oligomers, even though both involve keto C=O---Mg. We assume that the steric factors in oligomer formation are less favorable than those involved in dimer formation, resulting in keto C=O---Mg interactions of slightly lower average energy. In 10^{-2} M solutions in aliphatic solvents, the value of n in reaction 2 is at least 2 or 3.[22] Dry solutions of chlorophyll a in aliphatic hydrocarbon solvents contain appreciable concentrations of oligomers, and thus differ significantly from solutions of the same concentration in dry CCl_4.[22]

Equilibria 3, 4, and 5 describe the formation of chlorophyll dimer monohydrate, chlorophyll dimer dihydrate, and chlorophyll monomer monohydrate. These equilibria have not been studied separately, but the sum of 3, 4, and 5, reaction 6, has been studied by Rappaport[17] and by us. We have postulated two reactions, 4 and 5, in the conversion of $Chl_2 \cdot H_2O$ to $Chl \cdot H_2O$; water is first coordinated to form $Chl_2 \cdot (H_2O)_2$, and this species then disproportionates to $Chl \cdot H_2O$. This scheme, rather than the one-step reaction $Chl_2 \cdot H_2O + H_2O \rightleftharpoons 2Chl \cdot H_2O$ (reaction 6) is preferred because it appears to be more consistent with tetrahydrofuran titration data (Table I). The titration data indicates that disolvates are more stable in *n*-butylcyclohexane than in CCl_4, and thus two equilibria make it possible to differentiate between various solvents in a more precise way. Ir data, both in the carbonyl region and the OH stretch region, indicate that dissolution of solid chlorophyll $\cdot H_2O$ (1:1) in CCl_4 forms a solution containing free water and the dimer monohydrate, $Chl_2 \cdot H_2O$. Below concentrations of 0.01 M, the solutions contain $Chl_2 \cdot H_2O$ and $Chl \cdot H_2O$. (We have no way at present to distinguish between $Chl_2 \cdot H_2O$, $Chl_2 \cdot (H_2O)_2$, $Chl \cdot H_2O$, or $Chl \cdot (H_2O)_2$.) A study of the corresponding reaction with the base methanol, $Chl_2 + 2CH_3OH \rightleftharpoons 2Chl \cdot CH_3OH$, gave an equilibrium constant of 45 l. mol^{-1}; a value of 56 l. mol^{-1} was found in an earlier nmr study on chlorophyll–alcohol interactions.[7] For H_2O a value of ~100 l. mol^{-1} for K_6 was deduced by Rappaport.[17] Equilibrium 7 is not known to be important, probably because of the strictly limited solubility of H_2O in most nonpolar solvents. In CCl_4 or benzene, this equilibrium is clearly displaced to the left. It may, however, be important in very dilute chlorophyll solutions where a stoichiometric excess of water may be present.

Equilibrium 8 is the one in which the solvent characteristics play a decisive part. This reaction describes the formation of polymeric chlorophyll species in which $(Chl \cdot H_2O)$ is the repeating unit. The ir data gives evidence that this species contains water coordinated to magnesium, no free keto C=O, hydrogen-bonded carbomethoxy ester C=O, and hydrogen-bonded propionic ester C=O. This adds up to a structure in which water is coordinated to the central magnesium atom of one chlorophyll molecule, with the water in turn hydrogen bonded to the ester and keto carbonyl functions of another chlorophyll molecule. Repetition of the interaction then builds up a large aggregate or micelle. In CCl_4 solution, reaction 8 is displaced far to the left, and only in the most concentrated solutions can evidence be found for chlorophyll–water micelles. In aliphatic hydrocarbon solvents, however, reaction 8 is displaced, with what must be a large equilibrium constant, to the right. From ultracentrifugation data we judge n in reaction 8 to be very large, perhaps of the order of tens of thousands. Reaction 8 is forced to the right by decreasing the chlorophyll and increasing the water concentrations, and is reversed by raising the temperature or by increasing the chlorophyll concentration. Thus, a very dilute (10^{-6} M chlorophyll a) water-saturated solution in hexadecane shows only $(Chl \cdot H_2O)$ species, and no $(Chl \cdot H_2O)_n$ micelles, whereas in a 10^{-3} M solution, the $(Chl \cdot H_2O)$ occurs almost entirely as chlorophyll–water micelles.

Reaction 9 is included to account for a characteristic response of chlorophyll solutions in aliphatic hydrocarbon solvents to excess water. The addition of enough water to form a separate phase to a solution containing $(Chl \cdot H_2O)_n$ micelles results in the rapid formation of an insoluble precipitate, which by ir is the $(Chl \cdot H_2O)_n$ micelle fully hydrated by hydrogen bonding. If the water is removed, as by bubbling dry N_2 through the solution, the precipitate redissolves and finally a blue solution of chlorophyll dimers and oligomers is obtained.

The formation of chlorophyll–water micelles occurs with a large red shift in the visible absorption spectrum. Monomeric chlorophyll a absorbs at 665 nm, dimeric chlorophyll species absorb at 665 nm with a shoulder at 673 nm, and oligomeric chlorophyll a absorbs at 673 nm. Chlorophyll a–water micelles in hydrocarbon solvents absorb in the red at 740 nm. Monomeric, dimeric, and oligomeric chlorophyll a solutions are slightly different

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10^4 l. mol^{-1} for the monomer–dimer equilibrium constant in CCl_4, and more recently, Trosper and Sauer[36] gave the value $(4.4 \pm 1.1) \times 10^4$ l. mol^{-1} for the same constant. We have made new observations on the visible absorption spectrum of chlorophyll a in dry CCl_4 in an absorption cell that permits direct measurement of the absorption spectrum of a 0.1 M chlorophyll a solution in the visible region. When water and oxygen are rigorously excluded only slight differences can be detected between the spectra of a 0.1 M and a 10^{-6} M solution of chlorophyll a in CCl_4.[22] The spectrum of our dilute solution is almost identical with the dimer spectrum deduced by Sauer, *et al.*[35] This necessarily implies a considerably larger equilibrium constant for the monomer–dimer equilibrium than given by Sauer, *et al.*[35] As Sauer, *et al.*,[35] chose not to exclude water from their systems, there is good reason to suppose that they had present in their concentrated solutions Chl_2 and $Chl_2 \cdot H_2O$, and that the monomeric species present in their dilute solutions was $Chl \cdot H_2O$, and not Chl. Their difference spectra thus do not relate the monomer–dimer equilibrium.

(35) K. Sauer, J. R. L. Smith, and A. J. Schultz, *J. Am. Chem. Soc.*, **88**, 2681 (1966).

(36) T. Trosper and K. Sauer, *Biochim. Biophys. Acta*, **162**, 97 (1968).

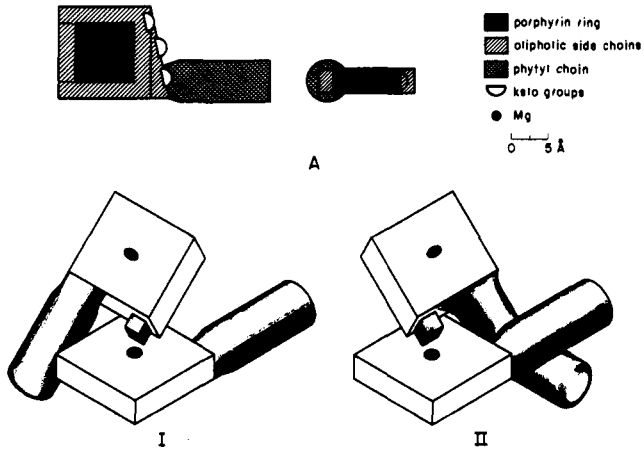

Figure 13. Dimer structures deduced from ir and nmr data. The dimensions are taken from Courtauld models. Types I and II represent limiting forms of dimer which are interconvertible by rotation.

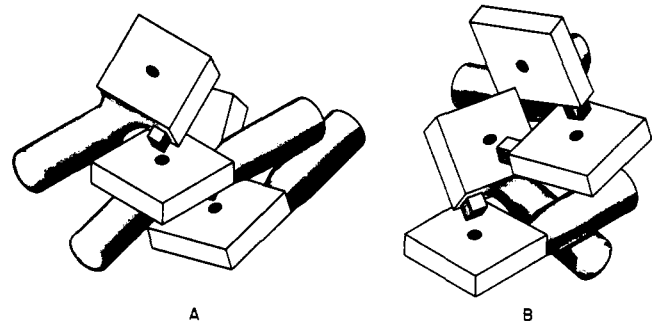

Figure 14. Oligomer structures derived (A) from type I dimer and (B) from type II dimer.

shades of blue; chlorophyll a–water micelles are yellow-green. Visible absorption spectra thus can be correlated with the presence of chlorophyll monomer, dimer, oligomer, and chlorophyll–water micelles.

The data now available do not permit an equally detailed discussion of the equilibria involving chlorophyll b, bacteriochlorophyll, or pyrochlorophyll a. For chlorophyll b, molecular weight data suggest that the fundamental unit in dry solutions is the trimer, $(\text{Chl b})_3$, that the aldehyde C=O groups are involved in the C=O---Mg interactions that form the dimer, which in turn uses one keto C=O group to form the trimer, leaving two keto C=O groups out of three free. The chlorophyll b equilibria are more strongly displaced in the direction of oligomer or chlorophyll b–water micelle formation than is the case for chlorophyll a. Thus, chlorophyll b–water micelles already form in $\sim 2 \times 10^{-2}$ M solutions in CCl_4, as judged from the red shift of the absorption spectra in the visible, and from the ir spectra. As for bacteriochlorophyll and pyrochlorophyll a, the evidence for formation of bacteriochlorophyll–water and pyrochlorophyll a–water micelles is also quite clear, but, structural details still remain to be established. The evidence indicates that the acetyl C=O group in bacteriochlorophyll does not coordinate to magnesium to a significant degree in the dimer or oligomer, and surprisingly appears to be even less strongly coordinated in the bacteriochlorophyll–water adduct. In pyrochlorophyll a–water adducts, the ir data indicates the persistence of keto C=O---Mg interactions even when excess water is present. Additional data will be required to establish the structure of the chlorophyll b–, bacteriochlorophyll–, and pyrochlorophyll–water adducts.

State of Water Coordinated to Mg. We have postulated strong hydrogen bonding between water coordinated to the magnesium of chlorophyll and the keto and ester carbonyl functions of another chlorophyll molecule. It is pertinent to point out, therefore, that the state of water coordinated to magnesium is such as to enhance its ability to form hydrogen bonds. We have noted elsewhere that nmr evidence indicates that water coordinated to magnesium is more acidic than bulk water.[19] The protons of water coordinated to the magnesium of chlorophyll experiences only a small diamagnetic shift from the macrocycle ring current, indicating that the protons are deshielded by coordination to Mg to an extent which compensates for the expected upfield shift.[7] Zundel and Murr[37] have likewise provided ir evidence that the charge distribution in the OH bonds of water coordinated to a cation is unusual. Thus, the OH stretching vibration of water coordinated to the Mg^{2+} of chlorophyll and hydrogen bonded to the C_9=O of a second chlorophyll molecule is shifted to 3220 cm^{-1} in solution and 3080 cm^{-1} in the solid from its normal value near 3700 cm^{-1}, indicative of a marked change in the OH bond. Coordination of water to cations leads to the formation of stronger hydrogen bonds,[37] and this must be an important factor in the stability of the $(\text{Chl}\cdot H_2O)_n$ micelles. The unusual electronic state of water coordinated to magnesium and hydrogen bonded to carbonyl functions may also have implications for photosynthesis, as it has been shown that photo-esr signals can be elicited from $(\text{Chl}\cdot H_2O)_n$ micelles by red light.[19]

Role of the Phytyl Chain. The chlorophyll phenomena described in this paper are strongly dependent on two factors: the presence of magnesium and an intact phytyl chain in the chlorophyll. We find that anhydrous methyl chlorophyllide a is almost insoluble in hydrocarbon solvents, and hydrated methyl chlorophyllide a does not form micellar solutions on ultrasonication as does chlorophyll a. Similarly, Sherman and Wang[16] found ethyl chlorophyllide a to behave very differently from the phytyl-containing chlorophyll a in Nujol solution. It seems reasonable to suppose that it is the energy of solvation of the phytyl chain by the hydrocarbon solvents that largely compensates for the loss in entropy in forming ordered oligomers or chlorophyll–water micelles. The ability of aliphatic or cycloaliphatic hydrocarbon solvents to solvate the dihydroporphyrin ring is so limited that in the absence of the phytyl moiety solubility is severely limited. Even though the energy of solvation of the phytyl must be small, it can be decisive because the difference in the free energy of formation of keto C=O---Mg and H_2O---Mg must be small.

The aggregation phenomena described in this paper depend on the presence of magnesium in the chlorophyll molecule. The ir spectra of the pheophytins do not

(37) G. Zundel and A. Murr, *Z. Physik. Chem.*, **54**, 49 (1967).

show the spectacular solvent dependence of the chlorophylls, and the pheophytin spectra are only slightly affected by the presence or absence of water. The loss of magnesium means that only π–π interactions can occur between pheophytin molecules, and coordination interactions of the kind discussed here cannot exist. Thus, pheophytins can in no way be considered as equivalent to the magnesium-containing chlorophylls.

Structure of Chlorophyll Dimers, Oligomers, and Chlorophyll–Water Adducts. The ir data indicate whether or not free keto groups exist in chlorophyll aggregates, whether C=O---Mg or C=O---HO(H)Mg interactions, or both, are involved, and whether the ester carbonyl groups are free or hydrogen bonded. A structural model must be compatible with the nmr data for chlorophyll a dimers, data which strongly suggest that overlap between dihydroporphyrin rings in the dimer is only partial, and that the overlapped regions in the two dihydroporphyrin rings are the same.[6,8] The nmr data also exclude configurations in which the phytyl chain is sited directly over a macrocycle ring, or in which the phytyl chain is wrapped around the periphery of a dihydroporphyrin ring in the plane of the ring. In the absence of any X-ray crystallographic data on bond distances or configuration in chlorophyll a, we take dimensions from Courtauld space-filling models.

With the above as boundary conditions, we suggest two possible configurations for chlorophyll a dimers (Figure 13A). These two structures appear to be compatible with both the ir and nmr data and are designated for convenience as types I and II. We judge type II to be somewhat the more stable because of the juxtaposition of the phytyl chains in this configuration. In the present context, perhaps the most important feature of these structures is that the dihydroporphyrin planes are not parallel, nor does there appear to be any way of making them so consistent with both the nmr and ir data.

Oligomers constructed from the two dimer types are shown in Figure 14. Again, these should be considered as limiting forms. In tetramer A constructed from two type I dimers, the phytyl chains point in two directions; in tetramer B constructed from two type II dimers, the phytyl chains are on one side only. Both tetramer structures are compatible with the ir data, but on an intuitive basis, the type B tetramer would seem to have the advantage in stability. It is apparent that while the two dimers I and II should be readily interconvertible, this could well not be the case for oligomers, and the equilibrium composition of a solution of oligomers might easily be a mixture constructed from the two basic dimer types. It appears clear, moreover, that none of these oligomer structures contain chlorophyll molecules with dihydroporphyrin rings parallel to each other. In any chlorophyll oligomer in which the bonding force is predominantly keto C=O---Mg interactions, the dihydroporphyrin planes cannot be made parallel. These considerations imply that efforts, such as those of Kreutz,[38] to apply Kasha-McRae[39,40] exciton theory

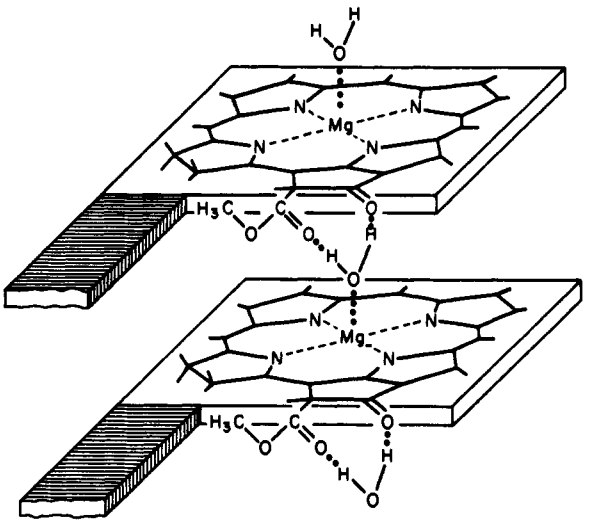

Figure 15. Structure illustrating the chlorophyll–water–chlorophyll interaction. The dimensions of the ring and the phytyl chain are not to scale.

to energy transfer in "aggregated" chlorophyll can scarcely be justified.

The structure of the chlorophyll–water micelle has been discussed elsewhere.[19] We show one of the repeating units in a chlorophyll–water micelle (Figure 15) to show the difference between this species and those shown in Figures 13 and 14. In this species the chlorophyll molecules easily can be made parallel, and such an arrangement is consistent with the very large shift from 665 to 740 mm observed in the absorption spectrum of this species.

It appears likely that the long-wavelength forms of chlorophyll so often reported in the literature[41] are chlorophyll–water adducts containing the repeating units of Figure 15. We have already shown that chlorophyll photo-esr signals require the presence of the structural entities of Figure 15.[19] We consider it highly probable that data in the literature on X-ray diffraction,[42,43] esr spectra,[43] infrared spectra,[16] aggregation and visible absorption spectra,[44] monolayers,[45] and photoconductivity[46] of chlorophyll will require reinterpretation in terms of the chlorophyll–chlorophyll and chlorophyll–water species described in this paper.

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