BBA 46277

CHLOROPHYLL-CHLOROPHYLL AND CHLOROPHYLL-WATER INTERACTIONS IN THE SOLID STATE*

K. BALLSCHMITER** AND J. J. KATZ
Chemistry Division, Argonne National Laboratory Argonne, Ill., 60439 (U.S.A.)

(Received August 27th, 1971)

SUMMARY

Absorption spectra in the visible and infrared have been recorded for films of dry chlorophyll *a*, chlorophyll *b*, bacteriochlorophyll, pyrochlorophyll *a*, and pheophytin *a*. The effects of water on the spectra have also been studied. From the infrared it is possible to deduce structures for the chlorophyll species involved, and to correlate the visible absorption spectra of both chlorophyll oligomers and chlorophyll-water adducts with particular chlorophyll species. Hydration may cause large red shifts in the visible absorption spectra, probably as a result of changes in the orientation of the chlorophyll molecules relative to each other.

INTRODUCTION

Chlorophyll in the plant is known to have unusual spectral characteristics[1]. Most prominent of these effects is a red shift in the absorption spectrum in the visible. It has long been recognized that long-wavelength forms of chlorophyll with similar spectral properties can be produced in the laboratory. The red shift in the visible absorption spectrum has almost invariably been attributed to chlorophyll "aggregation", and so it is not surprising that "aggregated" chlorophyll systems in the form of monolayers and films have been the object of considerable study[2]. It is reasonable to suppose that interpretation of the spectra of red-shifted *in vitro* systems should contribute to a better understanding of the state of chlorophyll in the plant. The prospects for now making such an interpretation on a molecular level, it would appear, have been considerably enhanced by new information derived from infrared and nuclear magnetic resonance (NMR) spectroscopy[3].

Chlorophyll in the solid state is particularly important for such studies because chlorophyll in this form mimics many of the properties of chlorophyll in nature. An extensive literature on the spectroscopy of "aggregated" chlorophyll exists[4-6], but no coherent picture for the molecular basis for the spectral shift has emerged. The earliest suggestion for the state of chlorophyll in the solid was advanced by

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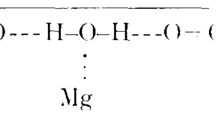
* Based on work performed under the auspices of the U.S. Atomic Energy Commission. Presented in part at the 5th International Conference on Photobiology, Hanover, N.H., August, 1968.

** Resident Research Associate, 1967-9; permanent address: Institut für Anorganische Chemie and Kernchemie, Johannes Guttenberg-Universität, 6500 Mainz, Germany.

HANSON[7] on the basis of X-ray diffraction studies in single crystals of ethyl chlorophyllide. In the light of more recent work it is probable that the crystals were those of a chlorophyll solvate. HANSON's portrayal of solid chlorophyll as consisting of parallel, staggered porphyrin planes was widely accepted and has since then governed proposed structures for "dimeric", "highly aggregated" or "crystalline" chlorophylls. This molecular packing, in fact, does not account for newer X-ray data available for chlorophyll *a*[8], and other data, particularly from NMR and optical rotatory dispersion studies[9], strongly imply that parallel orientations of the dihydroporphyrin planes cannot be achieved in pure chlorophyll.

Infrared[10] and NMR[11] data on chlorophyll can be interpreted on the basis that one or both axial positions of the central magnesium atom must always be filled with an electron donor molecule[12]. In the absence of extraneous nucleophiles, as in a nonpolar solvent or in a film of the solid, the coordination unsaturation of the central magnesium atom is satisfied by electron donation by the keto C=O function in Ring V of another chlorophyll molecule. The coordination interaction between the keto C=O function and the magnesium atom, referred to as keto C=O/Mg or C=O---Mg, leads to the formation of the dimer, Chl_2, in carbon tetrachloride or benzene solution, or to oligomers, $(Chl_2)_n$, with $n > 10$, in aliphatic hydrocarbon solvents[13]. The interaction between chlorophyll and water can also lead to the formation of large aggregates[14–16]; here aggregate formation results from water coordinated to the central magnesium atom of one chlorophyll molecule that is hydrogen bonded to the keto and ester C=O functions of another: keto  $\overline{C{=}O{-}{-}{-}H{-}\underset{\vdots \atop \mathrm{Mg}}{O}{-}H{-}{-}{-}O{=}C}$ ester. This interaction produces very large aggregates that are strongly red shifted[15].

Aliphatic hydrocarbon solvents provide a milieu especially conducive to the formation of chlorophyll oligomers and chlorophyll–water aggregates. Films may be thought of as limiting cases of solution in aliphatic hydrocarbon solvents, with the long aliphatic phytyl chains of the chlorophyll itself providing the hydrocarbon environment. Solutions, to be sure, make possible the achievement of dynamic equilibria. In films, to the contrary, molecular motions are much more constrained. Raising the temperature will increase the mobility of molecules in the film, as will the retention of solvent. But, in general, if a specific orientation is required for a particular interaction to occur, these will manifest themselves in a film only for those molecules that are correctly oriented relative to each other, or which require only small changes to bring them into the proper position. Phenomena in films thus may be expected to be very sensitive to the conditions under which the films are formed, and, especially important in the case of chlorophyll, will reflect the state of the chlorophyll in the solvent from which the film is cast. Thus, films prepared from carbon tetrachloride, where chlorophyll *a* exists predominately as the dimer, will have a smaller amount of keto C=O interactions than a film prepared from hexane, in which chlorophyll *a* occurs as an oligomer of larger size. The introduction of ligand molecules, such as water, into films may therefore be controlled by more complex factors than those involved when the interaction is carried out in a solution where the molecules are free to move about and to assume configurations of minimum energy. Whereas weak coordination interactions with specific steric requirements may be realized in solution, non-specific π-π interactions may be the only recourse available in films. Colloidal dispersions of hydrated chlorophyll in solvents such as Nujol appear to be intermediate between solutions and films, and although these mulls are often referred to as solids, they do not contain chlorophyll in the same state as in a true film[17].

In this paper we correlate the absorption spectra in the visible with infrared spectra in the O–H stretch region (3800–3000 cm^{-1}) and in the C=O stretch region (1800–1500 cm^{-1}) for films of anhydrous and hydrated chlorophylls *a* and *b*, pyrochlorophyll *a*[18], bacteriochlorophyll, and pheophytin *a*. The infrared data are used to make inferences about the chlorophyll species that give rise to the electron spectra. As in other investigations, we have carried out (T. M. COTTON, K. BALLSCHMITER AND J. J. KATZ, unpublished work, 1970), we find that the electronic spectra do not reflect structural changes in a highly sensitive way. Major changes in the structure of chlorophyll species as revealed by infrared may be accompanied by only small changes in the electronic spectra. Nevertheless, the correlations described here may be useful in drawing conclusions about the state of chlorophyll in the plant.

EXPERIMENTAL

Chlorophylls

These were obtained by the procedures of STRAIN AND SVEC[19]

Film preparation

Films of the dry chlorophylls were formed from carefully dried solutions in carbon tetrachloride, unless otherwise specified by drying in a stream of dry nitrogen. The thickness of the films was chosen experimentally to give absorbances between 1.3 and 1.5 in the visible region, and transmissions of 30–50 % for the strongest peak in the 1800-1500-cm^{-1} region in the infrared. For optical spectra, the chlorophyll films were cast in a 1-cm pathlength quartz cell that could be evacuated and connected to water vapor through a sidearm. For spectra in the infrared, the films were deposited on Irtran-2 plates, which could be pumped on or exposed to water vapor in a device similar to that used with the quartz cells. For examining the infrared spectrum under controlled water vapor pressure, a variable pathlength cell with Irtran-2 plates connected to a water reservoir was used. When it was desired to record both infrared and visible absorption spectra on the same preparation, BaF_2 discs were used. When the films have an absorbance of 1.7 in the visible, a scale expansion of 1.3–1.5 for scanning the infrared is advisable.

Hydration procedures

Two different procedures were used to study the effects of water on the spectra of chlorophyll in the solid state. In the first, dry chlorophyll solutions were hydrated either with liquid water or with water vapor in a hydrocarbon solvent to form chlorophyll hydrates which were used to form films. In the second procedure, a dry chlorophyll film was cast from carbon tetrachloride or hydrocarbon solution and the film was then exposed to water or water vapor.

In solution hydrations, about 2 mg of chlorophyll was thoroughly dried by codistillation with dry carbon tetrachloride or benzene as described elsewhere[21] and the dry chlorophyll dissolved in 0.2 ml cyclohexane. Under a N_2 atmosphere, 3–5 μl water were added, and the mixture sonicated at about 35° for several minutes. To insure the hydration of chlorophyll *b* and bacteriochlorophyll, heating to 45° for a short period to dissolve any unhydrated chlorophyll is recommended. The sonication was then repeated. The dispersions formed in this way were then used to cast the films for spectral examination. Another solution hydration procedure consists in allowing a $5 \cdot 10^{-2}$ M solution of dry chlorophyll in carbon tetrachloride to evaporate in an atmosphere saturated with water vapor at room temperature over a period of 40 to 60 h. This procedure, although time-consuming, appears to yield the best defined hydrates of chlorophyll *b* and bacteriochlorophyll[20].

Hydration of films is quite straightforward. The dry film is exposed to water vapor at the desired temperature. Vapor pressures below saturation were obtained by the use of saturated solutions of $MgCl_2$, KCl, or KOH in water.

Dehydration of hydrated chlorophylls was accomplished by pumping at 10^{-3}–10^{-4} mm Hg, or by heating to 70°, or by heating and pumping simultaneously.

All manipulations were carried out in a N_2-purged dry box in subdued light.

Spectra

Absorption spectra in the visible were recorded on a Cary 14 spectrophotometer, and infrared spectra on a Beckmann IR-7 instrument.

RESULTS

Infrared assignments are listed in Table I.

Chlorophyll oligomers

The infrared spectra of solid chlorophyll *a* have been studied by SIDEROV AND TERENIN[21], who reported that there were apparently no differences between concentrated solutions and films in the 3100–700-cm^{-1} region, but that a broad band is present at 3400 cm^{-1} in films. ANDERSON AND CALVIN[22] explained an observed shift from 1640 cm^{-1} (on AgCl plates) to 1655 cm^{-1} (KBr discs) by poorer "crystallinity" of chlorophyll *a* in the discs, defining "crystallinity" as the degree of parallel orientation of the porphyrin planes[23]. No systematic investigations on the infrared spectra of chlorophyll *b*, bacteriochlorophyll or pyrochlorophyll *a* in the solid state appear to have been carried out.

The O–H and C=O regions in the infrared of a film of anhydrous chlorophyll *a* cast from carbon tetrachloride is shown in Fig. 1A. There are no distinct bands in the OH region, and despite repeated attempts, we were unable to observe a band at 3495 cm^{-1} reported and assigned to the enolic OH of chlorophyll[21, 24, 25]. The very weak and broad band extending from 3200 to 3500 cm^{-1} probably is constituted of overtones of bands in the C=O region, as ^2H_2O exchange experiments show that this absorption does not involve exchangeable hydrogen[16].

The absorption spectrum in the carbonyl region is similar to that of anhydrous chlorophyll *a* dissolved in carbon tetrachloride and the assignments follow those made for the solutions[3]. Compared to the solution spectra in aliphatic hydrocarbons, where the solvent–solute interaction can be expected to be minimal, the absorption peak of the uncoordinated keto C=O in the solid is broadened and shifted from 1703 to 1695 cm^{-1}; the absorption peak of the magnesium-coordinated keto C=O is likewise

TABLE I

INFRARED SPECTRA (1800–1500 cm^{-1}) OF FILMS OF ANHYDROUS CHLOROPHYLL OLIGOMERS

		Propionic acid $C=O$	*Carbo-methoxy* $C=O$	*Free keto* $C=O$	*Free aldehyde* $C=O$	*Coordinated keto* $C=O/Mg$	*Coordinated aldehyde* $C=O/Mg$	*Skeletal* $(C=C)$ $(C=N)$
Chlorophyll *a*	$(Chl_2)_n$	1736	1736	1695	—	1655	—	1610
Chlorophyll *b*	$(Chl_3)_n$	1736	1736	1700	1633		1606	1606
Bacteriochlorophyll	$(BChl_3)_n$	1734	1734	1670		1645		1610, 1590, 1523
Pyrochlorophyll *a*	$(PyChl_2)_n$	1735	—	1687		1643		1655

broadened and shifted from 1660 to 1655 cm^{-1}. The ester C=O, however, is practically unaffected.

The relatively high intensity of the free keto C=O absorption maximum at 1695 cm^{-1} leads to the somewhat paradoxical conclusion that the extent of aggregation *via* keto C=O/Mg interactions in the solid film is considerably less than that found in concentrated ($>5 \cdot 10^{-2}$ M) solutions of chlorophyll *a* in aliphatic hydrocarbon solvents[13, 16].

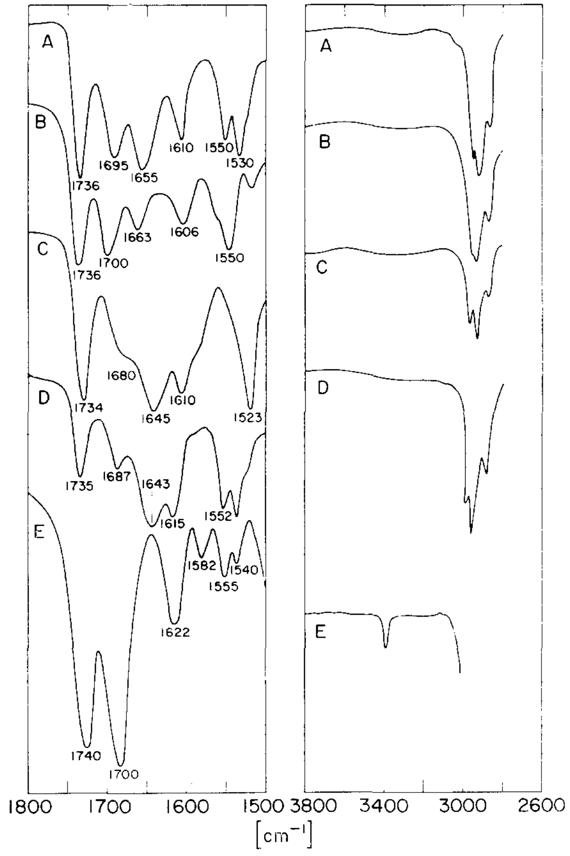


Fig. 1. Infrared spectra of films of anhydrous chlorophyll oligomers in the 1800–1500-cm^{-1} and 3600–2800-cm^{-1} regions. A, chlorophyll *a*; B, chlorophyll *b*; C, bacteriochlorophyll; D, pyrochlorophyll *a*; E, pheophytin *a*.

Chlorophyll *b* can be rendered anhydrous by co-distillation with benzene at 60–65° under a stream of dry nitrogen[20]. Anhydrous chlorophyll *b* is easily soluble in cyclohexane or *n*-butylcyclohexane at concentrations up to $5 \cdot 10^{-2}$ M. Ready solubility in cyclohexane can be taken as a measure of the anhydrous state of chlorophyll *b*. Traces of water quickly precipitate chlorophyll *b* from cyclohexane solution.

The additional carbonyl group in chlorophyll *b* complicates any interpretation of the infrared spectra in the C=O region, as in addition to a keto C=O/Mg, an aldehyde C=O/Mg coordination can also occur[11]. The occurrence of two "aggregation sites" for chlorophyll *b* and the results of molecular weight measurements[13] are at present best explained by the assumption that the basic unit of chlorophyll *b* in non-polar solvents at room temperature is a trimer. The chlorophyll *b*–chlorophyll *b* interaction has previously been generally treated[26–29] as a dimer interaction, and it is not clear now how the treatment of equilibria involving chlorophyll *b* or bacteriochlorophyll will be affected by the postulate of a basic trimer unit.

Fig. 1B shows the C=O region in the infrared of a film of anydrous chlorophyll *b* cast from benzene. There is no absorption in the O–H region. Normally the 1663-cm^{-1} band is considered to contain a contribution of a keto C=O coordinated to magnesium[3]. But considering the strong free keto C=O absorption at 1700 cm^{-1}, and the fact, that in pure, dry (alcohol-free) chloroform the whole 1663 cm^{-1} band is displaced to lower wave numbers as a result of hydrogen bonding of the solvent, it appears possible that the keto C=O/Mg interaction contributes only very little to the 1663-cm^{-1} absorption, as the C=O/Mg interactions should not be affected by the hydrogen bonding of the solvent chloroform.

Compared to the infrared spectrum of anhydrous chlorophyll *b* in an aliphatic hydrocarbon solution, the spectrum in the solid state shows (in the C=O region) a small shift to lower wave numbers for the free keto C=O (1707 to 1700 cm^{-1}) and the free aldehyde C=O (1672 to 1663 cm^{-1}), whereas the ester C=O and the band assigned to the aldehyde C=O/Mg interaction are not shifted. The relative intensities of the bands are unchanged in the solid compared to the solution in carbon tetrachloride[3] but differ strongly from a solution in *n*-butylcyclohexane, where the free keto C=O absorption is diminished[16]. This allows the conclusion, that, like chlorophyll *a*, chlorophyll *b* is less aggregated by keto C=O/Mg interaction in the solid than in a hydrocarbon solution, and will be present predominantly as trimer in the solid as judged from the similarity of the infrared spectra.

To our knowledge, no previous study of the infrared spectra of bacteriochlorophyll in the solid state have been reported. The acetyl carbonyl group present in position 2 of this chlorophyll increases the possibilities of coordinated interactions and the same general considerations with respect to possible aggregate structures as advanced for chlorophyll *b* can be made for bacteriochlorophyll. NMR studies[29] show that bacteriochlorophyll exhibits, as does chlorophyll *b*, two "aggregation sites", one near the keto C=O and the other in the vicinity of the acetyl carbonyl. As discussed below for chlorophyll *b*, this does not imply necessarily two different types of C=O/Mg interactions, but can result from structures involving only one type of C=O/Mg interaction. Whether bacteriochlorophyll favors the coordination number 5 or 6 for the magnesium atom, as chlorophyll *a* appears to do, is not yet determined. The assignments of the absorption in the infrared follow previous studies of solution spectra[3, 16], but they have some ambiguity due to unresolved maxima in the C=O region.

Bacteriochlorophyll can be rendered anhydrous by co-distillation of the water with benzene at 60–70°, under a stream of dry nitrogen[16]. Anhydrous bacteriochlorophyll is easily soluble in dry cyclohexane, but precipitates when traces of water are present. Solubility in cyclohexane can be taken as confirmation of the anhydrous state.

Fig. 1C shows the C=O and O–H stretch regions in the infrared of a film of anhydrous bacteriochlorophyll cast from dry carbon tetrachloride. In the O–H region, there is a very weak and broad absorption centered at about 3350 cm^{-1}. The absorption band of the free acetyl C=O at 1660 cm^{-1} has completely disappeared due to overlapping, as has been observed for higher aggregates in solution[16]. There is no clear evidence as to whether the band at 1610 cm^{-1} is to be assigned to an acetyl C=O/Mg interaction or to skeletal vibrations only. The "aggregation site" at the acetyl carbonyl as found by NMR[26] could be explained by a keto C=O/Mg interaction with overlapping of "keto" and "acetyl" parts of the molecules involved.

Compared to anhydrous bacteriochlorophyll in *n*-butylcyclohexane[16] solution, only the absorption of the free keto C=O is broadened and shifted in the solid (from 1700 to 1680 cm^{-1}) and as is the case for chlorophyll *a*, this shift is somewhat larger than that observed for the change from *n*-butylcyclohexane to carbon tetrachloride solution (from 1703 to 1695 cm^{-1}).

Pyrochlorophyll *a* is a valuable substance for aggregation studies of chlorophyll *a*, as it allows conclusions about the way the bulky $-O-CO-CH_3$ group at Ring V affects the C=O/Mg interaction of the C_9-ketocarbonyl function. The assignments for this substance follow previous infrared studies of this compound[3,16,18].

Pyrochlorophyll *a* can be made anhydrous by heating a film cast from carbon tetrachloride to 60–70° for several hours in a high vacuum[16]. Fig. 1D shows the C=O region in the infrared of a film of anhydrous pyrochlorophyll *a* cast from cyclohexane. The weak absorption of the free keto carbonyl can be taken to indicate a large amount of C=O/Mg interaction, which will be sterically favored, compared to chlorophyll *a*, by the lack of the carbomethoxy group.

Dry pheophytin *a* (Fig. 1E) shows no evidence for keto C=O interactions in the infrared, entirely as expected.

The absorption spectra in the visible of all of the chlorophyll oligomers are treated below.

Chlorophyll a hydrates

In this section the infrared spectra of films of chlorophyll hydrates will be described. KARYAKIN AND CHIBISOV[30] and KARYAKIN *et al.*[24] investigated the effects of water on films of mixtures of chlorophylls *a* and *b*. Their results were interpreted on the basis that chlorophyll *a* occurs as an enol in the solid state[31] an assumption now open to serious question[16]. SIDEROV[32] studied ^2H_2O exchange with films of chlorophyll *a* and pheophytin *a* by infrared, but surprisingly reported no differences in the C=O region between anhydrous and hydrated chlorophyll *a*. SHERMAN AND WANG[33,34] made a valuable study of the effect of water on chlorophyll *a* and ethyl chlorophyllide *a* in Nujol mulls by infrared spectroscopy, but did not advance an explanation for their results. Hydration of anhydrous chlorophyll *a* in cyclohexane solution with a little water for 20 min at about 35° yields the $(Chl \cdot H_2O)_n$ species that has been described elsewhere[16]. The infrared spectra of a film (Fig. 2A) resemble, but are not identical, with the spectra observed for this species in the form of a colloidal dispersion in an aliphatic hydrocarbon solvent. The O–H region of a hydrated film shows a broad, undefined band with a maximum near 3080 cm^{-1}. The band at 3080 cm^{-1} is assigned to O–H stretch of water coordinated to magnesium and hydrogen bonded to the keto C=O. A sharp band at 3610 cm^{-1} sometimes seen is probably the free O–H vibration of water, which is present at 3710 cm^{-1} and 3615 cm^{-1} in carbon tetrachloride solution. In the C=O region, free keto C=O near 1700 cm^{-1} is very weak, but the peaks characteristic of this species at 1640 cm^{-1} is very strong. The most important spectral difference in the film is the absence of ester C=O splitting. This implies that the restricted mobility of the chlorophyll in the film prevents the formation of hydrogen bonds to the carbomethoxy C=O function as is the case in solution. In agreement with this, the electronic properties of films of this species are detectably different.

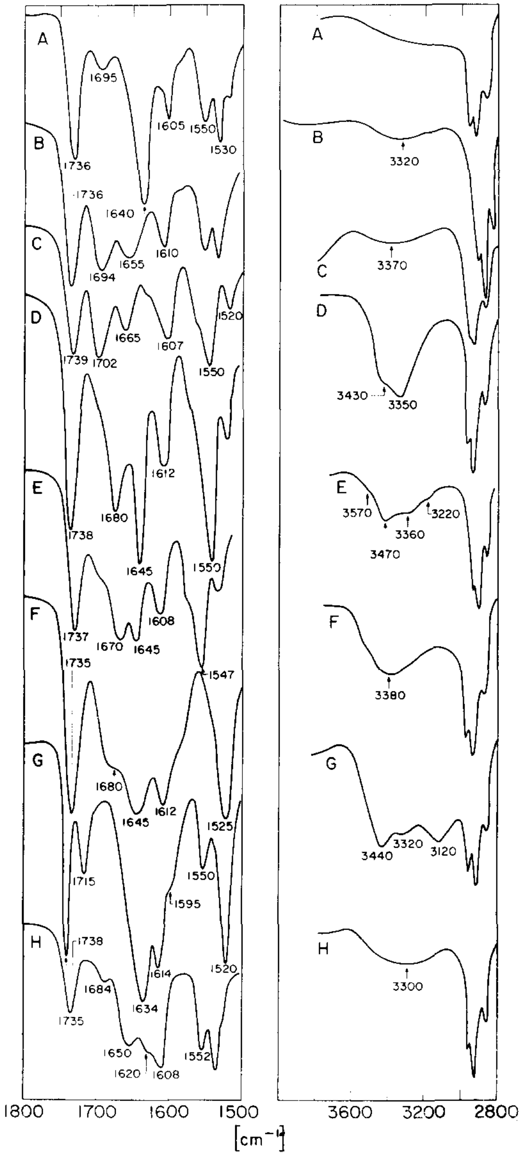


Fig. 2. Infrared spectra of films of hydrated chlorophyll species in the 1800–1500-cm^{-1} and 3600–2800-cm^{-1} regions. A, (Chl $a \cdot H_2O)_n$; B, (Chl $a)_2 \cdot H_2O$; C, (Chl $b)_3 \cdot H_2O$; D, (Chl $b \cdot H_2O)_n$, the "stable" hydrate; E, (Chl $b \cdot H_2O)_n$, the "unstable" hydrate; F, (BChl)$_3 \cdot 2H_2O$ or (BChl)$_3 \cdot H_2O$; G, ((BChl)$_3 \cdot H_2O)_n$; H, (pyrochlorophyll $a \cdot H_2O)_n$.

When a film of $(Chl \cdot H_2O)_n$ prepared as above is pumped on for 24 h at 10^{-3} mm Hg and room temperature, water is displaced from magnesium and C=O/Mg interactions are regenerated to form the species, $Chl_2 \cdot H_2O$. The O–H and C=O regions in the infrared for the chlorophyll *a* dimer monohydrate are shown in Fig. 2B. In the OH region a weak and broad band with the maximum at 3320 cm^{-1}, extending from 3200 to 3500 cm^{-1}, is observed. The maxima in the C=O region are the same as for anhydrous chlorophyll (Fig. 1A), but the relative intensities are different. The band at 1694 cm^{-1}, assigned to the free keto C=O is stronger, indicating that less C=O/Mg interaction occurs in the hydrated film than in the anhydrous state. This is consistent with a structure for the dimer monohydrate in which one magnesium atom has the coordination number 5 by interaction with the keto C=O of a second chlorophyll molecule, which, in turn, has one water molecule coordinated to its magnesium atom.

The O–H and C=O region do not tell much about hydrogen bonding of the coordinated water, although any interaction with the carbonyl groups as hydrogen bond acceptors can be excluded because no spectral shifts are found in the C=O region. Other possibilities of hydrogen bonding are discussed in detail later for the chlorophyll solvate 3Chl $b \cdot 2H_2O$.

Chlorophyll b hydrates

KARYAKIN *et al.*[24] studied the interaction of water with films of chlorophyll *b* by infrared spectroscopy and found a broad band at 3600–3200 cm^{-1}, whose intensity did not change when the film was heated to 55° in a high vacuum (10^{-5} mm Hg) for 1.5 h. No changes were observed in the C=O region upon pumping in a high vacuum. A recent infrared study of chlorophyll *b*–chlorophyll *b* and chlorophyll *b*–water interactions in solution[16] has revealed some new aspects about the molecular interactions in these cases.

Anhydrous chlorophyll *b* is strongly hygroscopic and readily forms hydrates with compositions intermediate between 3:2 and 3:1. This is consistent with other evidence that indicates the fundamental chlorophyll *b* unit to be the trimer. Fig. 2C shows the infrared spectra of films of these species. In the O–H region, a strong, broad band with a maximum at 3370 cm^{-1} occurs. The C=O region is very nearly the same as in anhydrous chlorophyll *b* (Fig. 1B) as regards the position of maxima and the relative intensities of the bands. The bands at 1702 and 1607 cm^{-1}, however, are sharper. The bound water, which cannot be removed by heating to 60° and pumping for 4 h at 10^{-3} mm Hg, thus cannot be involved in hydrogen bonding to C=O functions, nor does it strongly affect any C=O/Mg interactions, although the slight increase in the intensity of the free keto absorption at 1702 cm^{-1} suggests that some disruption of keto C=O/Mg interaction has occurred. It appears reasonable to us to coordinate the water molecules to the magnesium atoms of the chlorophyll *b* trimer: $H_2O \cdot$Chl *b* Chl *b* Chl $b \cdot H_2O$, as at least two of the magnesium atoms of the trimer have the coordination number 5. SIDEROV AND TERENIN[21] have postulated hydrogen bonding of water to the nitrogen atoms of the pyrrole rings, but this appears to us a less likely possibility. It remains a possibility, however, that a water molecule coordinated to the magnesium atom of a trimer might, because of its enhanced acidity, coordinate to the nitrogen atoms of another trimer, or even with the pyrrolic nitrogen of the chlorophyll molecule to which it is attached by coordination to magnesium; in this event, it might be expected that the pyrrolenic nitrogen of Ring IV might be involved. The infrared data seem to indicate clearly that interaction of water with the aldehyde C=O function of chlorophyll *b* to form structures similar to chloral hydrate is not important; this should decrease the aldehyde C=O absorption intensity, which, however, is not the case.

By more vigorous hydration conditions, water molecules can be inserted between the chlorophyll molecules of the trimer to form chlorophyll *b* species that appear formally analogous to the (Chl $a \cdot H_2O)_n$ species. The situation with chlorophyll *b*, however, is much more complex because of the additional hydrogen bonding possibilities presented by the additional C=O group present in the aldehyde function of chlorophyll *b*. To complicate matters further, it appears that two chlorophyll *b* hydrates can be formed, one "stable" in the sense that the incorporated water cannot readily be removed, the other "unstable" in that exposure to infrared radiation causes reversion to the anhydrous form. Although the infrared spectra are not identical for the two forms, the structural basis for the difference in stability is still obscure.

The "stable" chlorophyll *b* monohydrate is prepared by shaking a solution of chlorophyll *b* in carbon tetrachloride at a concentration of $> 5 \cdot 10^{-2}$ M with a little water, or allowing such a solution to evaporate in a water-saturated atmosphere over a period of 40–60 h. Direct analysis[20] indicates the composition Chl b:H_2O of 1:1. The chlorophyll *b* monohydrate is dispersed in water-saturated cyclohexane by ultrasonication, and films are formed by evaporation of the solvent. The "unstable" hydrate can be prepared by exposure of a chlorophyll *b* film at 55° to water vapor for 70 h, or by hydration with excess water in cyclohexane solution. The conditions for the preparation of the two species are not very different, and the factors that determine which species is formed are not clearly understood. It would appear, however, that the differences are associated with the state of the chlorophyll *b* being hydrated. The more highly aggregated the chlorophyll *b* (*i.e.* cyclohexane *vs.* carbon tetrachloride) the more likely the formation of an "unstable" adduct. The infrared spectra of the two chlorophyll *b* hydrates are shown in Figs. 2D and 2E.

We discuss the "stable" chlorophyll *b* hydrate first. In the O–H region, two strong bands are observed at 3430 and 3350 cm^{-1}. In the C=O region there are absorption peaks at 1738, 1680, 1645, 1612 and 1550 cm^{-1}. Compared to the "unstable" hydrate, the "stable" chlorophyll *b* hydrate lacks the maxima at 3570 and 3220 cm^{-1} in the O–H region and the band at 1670 cm^{-1} in the C=O region. However, there is a band at 1680 cm^{-1} and the absorption at 1645 cm^{-1} is very strong.

In a previous study[16] the 1680-cm^{-1} band has been assigned to the free aldehyde C=O, which appears at 1665 cm^{-1} in carbon tetrachloride solutions of anydrous chlorophyll *b*. (This peak is found at 1672 cm^{-1} in *n*-butylcyclohexane in what appears to be a solvent effect.) The band at 1645 cm^{-1} was assigned to the keto C=O/H_2O/Mg interaction as postulated for chlorophyll *b*. Because the two chlorophyll *b* monohydrates, the "stable" and the "unstable" have a spectrum in the O–H and the C=O region which is characteristically different, two different molecular organizations must be involved.

For the "stable" chlorophyll *b* monohydrate formed by hydration in carbon tetrachloride solution, we advance the structure shown in Fig. 3. The assignments are different from those previously made[16]: 1680 cm^{-1}, keto C=O/H_2O/Mg; 1645 cm^{-1}, aldehyde C=O/H_2O/Mg. The water is coordinated to the magnesium atom and its two O–H vibrations are involved in hydrogen bonding with the keto C=O (3430 cm^{-1}) and the aldehyde C=O (3350 cm^{-1}) of two different chlorophyll *b* molecules.

The relatively small shift in the frequency of the keto C=O upon hydrogen bonding to water coordinated to magnesium (approx. 20 cm^{-1}) in chlorophyll *b* compared to the same interaction of the keto C=O in chlorophyll *a* (shift approx. 40 cm^{-1}) may be explained by a somewhat longer distance between the interacting molecules. According to the X-ray pattern, a Mg–Mg spacing of 7.6 Å is found in the chlorophyll $a \cdot H_2O$, whereas the analogous spacing is 10.0 Å in the chlorophyll *b* monohydrates[20].

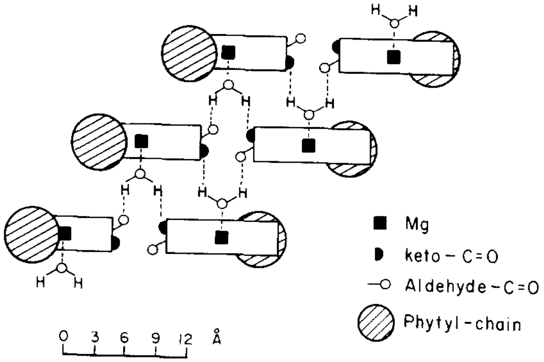


Fig. 3. Possible structure for stable chlorophyll *b* monohydrate adduct $(\mathrm{Chl}\ b \cdot H_2O)_n$. Dimensions taken from space filling Courtauld models.

The "unstable" chlorophyll *b* hydrate in the O–H region possesses a shoulder at 3570 and maxima at 3470, 3360 and 3220 cm^{-1}. Even more distinct changes appear in the C=O region of this hydrate compared to the 3:2 hydrate. The strong band at 1700 cm^{-1} of the free keto C = O is greatly diminished and a new maximum at 1645 cm^{-1} appears. Other maxima appear at 1670 and 1608 cm^{-1}.

The disappearance of the absorption for the free keto C=O together with the new absorption peak at 1645 cm^{-1} is tentatively assigned to the formation of a $C=O/H_2O/Mg$ interaction as described for chlorophyll *a*[16].

This necessitates the assignments of the 1670-cm^{1-} absorption peak to the free aldehyde C=O and the 1608 cm^{-1} absorption to C=O and/or C=N skeletal vibrations. But it is difficult to make the observed maxima in the O–H region consistent with this model for the chlorophyll *b*–water interaction, because the observed four maxima in the O–H region require, as a first approximation, four different types of hydrogen bonded O–H vibrations. The absence of any ester splitting in the C=O region excludes further hydrogen-bonding assignments, unless it is assumed that in the solid state, hydrogen bonding to ester C=O is not reflected in a shift of its absorption peak, which does not appear likely.

On standing in the infrared spectrophotometer, a film of chlorophyll *b* "cyclohexane hydrated" undergoes pronounced spectral changes in the visible and in the infrared (Fig. 2E), which are a result of the loss of water. The correlation of changes in the C=O region with that in the O–H region helps only a little to clarify the nature of this chlorophyll *b*–water interaction. The loss of the 1645-cm^{1-} absorption, the increase of the 1695- or 1702-cm^{-1} absorption and the shift of the 1670-cm^{-1} band to 1665 cm^{-1} in the C=O region seem to parallel the loss of the 3570-cm^{-1} shoulder and the decrease at 3470 and 3220 cm^{-1} in the O–H region, finally showing a maximum at 3360 cm^{-1} as observed with the chlorophyll *b* (3:2) hydrate.

We do not have enough data on the "unstable" chlorophyll *b* hydrate to suggest a structure. It would appear plausible that the "unstable" hydrate is formed by insertion of water into chlorophyll *b* oligomers in such a way that minimal reorganization of the oligomer occurs. This would make it readily possible to reconstitute carbonyl–magnesium interactions by expulsion of water, and thus would contribute to the thermal instability of this hydrate.

Bacteriochlorophyll hydrates

If water is not strictly excluded, bacteriochlorophyll forms a hydrate, which according to direct water analysis by a recently described gas chromatographic procedure[20] has a mole ratio of 3:2–3:1. Fig. 2F shows the O–H and the C=O region in the infrared of this hydrate. Except for the strong and broad band in the O–H region (3380 cm^{-1}) the spectra are identical with the anhydrous bacteriochlorophyll and the assignments are the same.

The broad band at 3380 cm^{-1} can best be explained by strongly hydrogen-bonded water, though there is no evidence for an involvement of the carbonyl groups, which most likely would serve as the acceptors for the hydrogen bond. We, therefore, advance the same pigment–water interaction as discussed for the chlorophyll *b*-water (3:2) species, with the water coordinated to the magnesium atom and hydrogen bonded either to the pyrrole nitrogen of the same molecule or to a second one.

Hydration of a solution of bacteriochlorophyll in cyclohexane with excess of water results in a bacteriochlorophyll hydrate with drastically changed spectral properties. This product is stable at 25° in a high vacuum. The pigment-water ratio has not yet been determined, but is seems likely that it is a monohydrate, as found for chlorophyll *a* and *b* under the same conditions.

Fig. 2G shows the O–H and C=O region in the infrared of a bacteriochlorophyll hydrate prepared by the above described procedure. The O–H region shows three distinct absorption maxima at 3440, 3320, and 3120 cm^{-1}. The C=O region has also changed drastically compared to the anhydrous bacteriochlorophyll and maxima are found at 1738, 1715, 1634, 1614, 1595, 1550 and 1520 cm^{-1}. The 1735-cm^{-1} ester band in anhydrous bacteriochlorophyll has split into two maxima at 1738 and 1715 cm^{-1}, the maxima at 1680 and 1645 cm^{-1} are replaced by a very intense band at 1634 cm^{-1}, the band at 1525 cm^{-1} has split into a band at 1550 and at 1520 cm^{-1}, respectively. With bacteriochlorophyll it is the first time that an ester splitting is observed in the solid; with chlorophyll *a* it only appears in colloidal solutions of the monohydrate[16,17], and with chlorophyll *b* has not been observed at all.

We assign the disappearance of the free keto carbonyl at 1680 cm^{-1} and the keto C=O/Mg interaction at 1645 cm^{-1} together with the new band at 1634 cm^{-1}, to a Mg/H_2O/C=O interaction between two molecules of bacteriochlorophyll[16].

The absence of a C=O absorption band which can be assigned to the free acetyl C=O may be explained by the superposition of the very strong 1634-cm^{-1} band.

In the 3800–3000-cm^{-1} region we assign the band at 3120 cm^{-1} to an O–H stretch vibration of water coordinated to magnesium and hydrogen bonded to the keto C=O, the band at 3440 cm^{-1} to an O–H stretch vibration of water coordinated to magnesium and hydrogen bonded to the ester C=O of the carbomethoxy ester group, resulting in its shift in the C=O region from 1737 to 1715 cm^{-1}.

These assignments leave the question open of the origin of the 3320-cm^{-1} absorption in the O–H region. For chlorophyll *a* and *b* this absorption band cannot be assigned unambiguously. It may be the O–H absorption of occluded water, which is not lost upon casting the film or on heating the film to 60°. The loss of the 3440- and 3120-cm^{-1} absorption upon heating then would be due not to the actual loss of water but only due to a change in its interaction with the chlorophyll. This should affect, of course, its absorption maxima in the O–H region.

Pyrochlorophyll a hydrates

Pyrochlorophyll *a* can be hydrated either as a film, by exposure to water at 55°, or in cyclohexane by shaking with water[16]. The infrared spectra of hydrated pyrochlorophyll *a* prepared in either way are identical (Fig. 2H). The stoichiometry of the pyrochlorophyll *a*–water interaction has not yet been established. Upon hydration, the spectrum in the 1800–1550-cm^{-1} region in the infrared changes quite drastically; the absorption of the free keto C=O at 1683 cm^{-1} has diminished, while the absorption at 1643 cm^{-1} assigned to the keto C=O/Mg interaction has split into two maxima at 1650 and about 1620 cm^{-1}, the latter being overlapped by the 1608-cm^{-1} band. Following a previous study[16] we assign the maximum at 1620 cm^{-1} to the keto carbonyl of a C=O/H_2O/Mg interaction between dimers of pyrochlorophyll *a* held together by a C=O/Mg interaction, which results in the 1650-cm^{-1} band. The 1608-cm^{-1} absorption we assign to a C=O and/or C=N skeleton vibration.

The absorption in the O–H region in the infrared shows only a broad band with the maximum at 3300 cm^{-1} and permits no other specific assignments other than the water is strongly hydrogen bonded.

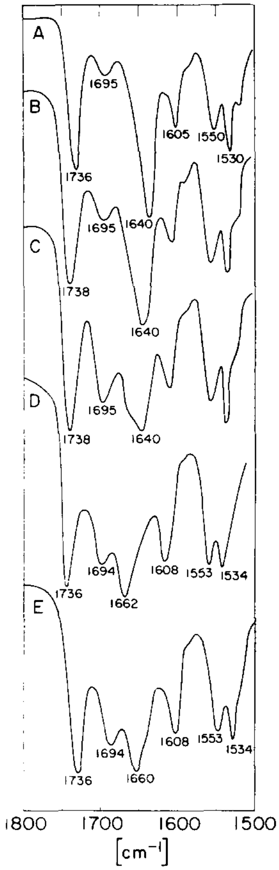
Hydration of chlorophyll a films

When chlorophyll is hydrated in solution, the chlorophyll molecules are free to assume configurations of maximum stability. Hydration of films, where the mobility of the chlorophyll is restricted, must reflect to a considerable extent the orientation of the chlorophyll molecules in the particular film, and this, in turn, will be greatly affected by the solvent, concentration, rate of evaporation and other experimental conditions that are not easily controlled. Film phenomena are thus of lesser intrinsic interest, but nevertheless are quite informative about events involved in the interactions of chlorophyll and water.

To make a quantitative comparison of the changes in the infrared spectrum (Fig. 4A) of a film of chlorophyll *a* upon hydration, a film of anhydrous chlorophyll *a* cast from carbon tetrachloride was exposed at 53° for 30 min to water vapor at about 100 mm Hg. (Fig. 1A shows the absorption maxima of the anhydrous chlorophyll *a* film in the O–H and C=O regions.) Additional hydration prolonged for another 24 h did not change the infrared spectrum further.

The O–H region shows an unstructured absorption extending from 3000 to 3600 cm^{-1} (Fig. 5A). No particular assignments can be made other than that the water is strongly hydrogen bonded. In the C=O region, however, distinct changes upon hydration are observable. There is little change in the position of the ester band at 1736 cm^{-1}, the absorption of the keto C=O at 1695 cm^{-1} is strongly diminished, and the band at 1655 cm^{-1} is replaced by a strong absorption at 1640 cm^{-1}. The band at 1610 cm^{-1} assigned to C=C and/or C=N skeletal vibrational decreases somewhat in intensity and shifts to 1605 cm^{-1}. Minor changes also occur in the bands at 1550 and 1530 cm^{-1}.

The new strong band at 1640 cm^{-1} we assign to a keto C=O/H_2O/Mg interaction, as in solutions[14,16]; this assignment also explains the decrease in the intensity of the keto C=O band at 1695 cm^{-1}. No splitting of the ester carbonyls can be observed in films, whereas it can in mulls[31,34] and in colloidal solutions of $(\mathrm{Chl}\cdot H_2O)_n$[16]. With the absence of ester splitting is the observation that in the O–H region none of the three distinct bands seen in Nujol mulls[16,31,34] can be observed.



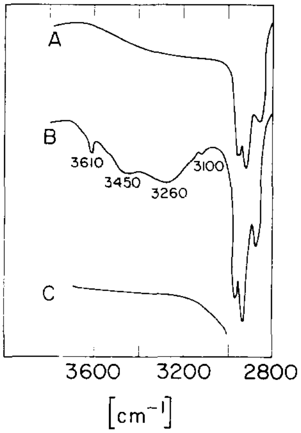


Fig. 4. Effect of hydration conditions on infrared spectra of chlorophyll *a* films in the 1800–1500 cm^{-1} region. A, film hydrated at 55° and a water vapor partial pressure of 100 Torr; B, film hydrated at 23° and 22 Torr partial pressure of water; C, effect of heating on (B); D, film hydrated at 23° and 7 Torr partial pressure of water; E, film hydrated by liquid 0.01 M phosphate buffer solution (pH 6.9).

Fig. 5. Effects of hydration conditions on the infrared spectra of chlorophyll *a* films in the 3600-2800-cm^{-1} region. A, B, and C are as shown in the legend for Fig. 4.

Chlorophyll *a* hydrated at lower temperatures and water vapor pressure yields a quite different infrared spectrum, and the hydrate that forms is unstable. Fig. 4B shows the C=O and Fig. 5B the O–H region of a film of chlorophyll *a* hydrated at 23° and 20 mm Hg for 40 h. The film originally had been cast from a dry carbon tetrachloride solution. Surprising is the structured absorption in the O–H region with maxima at 3610, 3450 and 3260 cm^{-1}, which probably represent distinct varieties of O–H vibrations in a pigment–water adduct. The possibility that an external water film contributes can be ruled out, as its absorption is quite different in the O–H region, and it would be expected to have no effect on the C=O region of the film. The shape of the absorption curve of a surface water film on the film of chlorophyll *a* resembles strongly the infrared spectra of the O–H region reported by several other authors[21, 24, 30] and which has been assigned to a water molecule hydrogen bonded to the two nitrogen atoms of the pyrrole rings of chlorophyll *a*; the assignment is made on grounds of a similar line shape of water hydrogen bonded to pyridine[21].

The structure of the less stable chlorophyll *a* hydrate (Fig. 6) prepared under milder hydration conditions can be derived from consideration of the orientation of the chlorophyll molecule prior to the incorporation of water. In ref. 16 we discuss two basic types of dimer (oligomer) orientation, which would result (without a general reorientation of the molecules, which is unlikely in the solid) in two different structures for hydrated chlorophyll films.

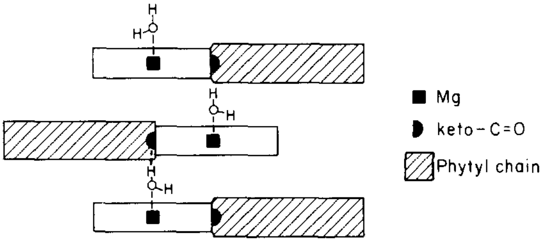


Fig. 6. Proposed structure of the chlorophyll *a*–water adduct (Chl $a \cdot H_2O)_n$ obtained by the hydration of chlorophyll *a* films.

We tentatively assign the 3450- and 3260-cm^{-1} bands (Fig. 5B) to water coordinated to magnesium hydrogen bonded to the keto C=O, as the absence of ester splitting in the C=O region can be interpreted to indicate that the carbomethoxy group does not function as a hydrogen bond acceptor. Whether one keto C=O is the acceptor of both the O–H interactions or whether the two O–H stretch vibrations reflect two different positions of the water cannot be decided. The appearance of the absorption of the free keto C=O at 1695 cm^{-1} and the decrease of the strong band at 1640 cm^{-1} leads to the conclusion that the keto C=O is involved in the hydrogen bonding.

Because the orientation of the chlorophyll molecules as present in the oligomers in solution will tend to be preserved in the solid film, the water should be easily driven out by the restoration of the C=O...Mg interaction. This is clearly the case for the chlorophyll hydrate prepared at 23°. Fig. 5C presents the changes in the O–H and C=O region, with time when such a chlorophyll *a* hydrate is kept for about 90 min in the sample compartment of the Beckmann IR 7 with the infrared source turned on. These data show clearly the correlation of the changes in the O–H and C=O region. With the loss of the 1640-cm^{-1} absorption and the growing in of the 1655-cm^{-1} band, the maxima at 3610, 3450, and 3260 cm^{-1} disappear in the O–H region.

If the vapor pressure of the water is still further reduced, the course of hydration is again different. Thus, SHERMAN AND LINSCHITZ[25] reported a red shift in the red band of the electronic absorption spectrum of chlorophyll *a* in a Nujol "mull" upon hydration at a water vapor pressure below 10 mm Hg; BALLSCHMITER AND KATZ[16] found that a specific chlorophyll–water interaction involving hydrogen bonding to the ester C=O is connected with this red shift.

Similar changes can be observed when films of anhydrous chlorophyll cast from carbon tetrachloride are exposed to a water vapor pressure between 5 and 12 mm Hg. Fig. 4D shows the change in the 1800–1500-cm^{-1} region in the infrared after hydration at 23° and a water vapor pressure of about 7 mm Hg (satured $MgCl_2$ solution) for 42 h. The absorption band of the ester C=O at 1736 cm^{-1} is slightly decreased with an increase of the absorption at 1717 cm^{-1}, due to hydrogen bonding of water to the ester carbonyl. The absorption band of the free keto C=O at 1694 cm^{-1} decreases markedly and the band at 1655 cm^{-1} sharpens with the maximum shifted to 1660 cm^{-1}. Within 5–10 min in the sample compartment of the Beckman IR 7, the spectrum of the anhydrous chlorophyll *a* film is regained (Fig. 1A), with a slight increase of the absorption of the uncoordinated keto C=O and a small decrease in the intensity of the C=O/Mg interaction.

It can be judged from the absence of any increase in absorption at 1640 cm^{-1} and from the electronic absorption spectrum, which shows no absorption at 740 nm, that the hydration at low P_{H_2O} did not lead to a C=O/H_2O/Mg interaction. The spectral changes are explained best by hydrogen bonding of water to the keto C=O resulting in the decrease at 1694 cm^{-1} and in the next maximum at 1660 cm^{-1} superimposed on the 1655 cm^{-1} absorption of the C=O---Mg. From the slight decreases at 1655 cm^{-1} and the increase at 1695 cm^{-1} after drying in the Beckman IR 7, it can be concluded that some of the C=O/Mg interaction must have been interrupted, although no C=O/H_2O/Mg interaction is formed. It is possible that the dimer units are not interrupted, but that water is inserted between the dimers, and that for steric reasons a C=O/H_2O/Mg interaction between dimers is not allowed. The changes at the 1534-cm^{-1} band have not yet been interpreted. Spectral changes in the visible accompany the changes in the infrared as water is lost from the film. The red band in the electronic transition spectrum loses a tailing to the red, the Soret band increases in absorbance, a difference maximum appears at 710 nm as loss of water proceeds. The spectral changes observed by KE AND SPERLING[35] on drying monolayers of chlorophyll *a* were interpreted by them as the result of a loss of order as water leaves the film.

The same effects in the infrared spectrum as described in Fig. 4D can be observed when a film of anhydrous chlorophyll *a* is covered by a 0.01 M phosphate buffer solution (pH 6.9) for 18 h (Fig. 4E). As in Fig. 4C, the spectrum had begun to change back to the "dry" state after 15 min in the Beckman IR 7.

The fact that the spectral consequences of hydration by a buffer solution is equivalent to those observed at 7 mm Hg water vapor pressure and not to that of 20 mm Hg may at first be surprising. But since chlorophyll *a* is insoluble in water, the tendency for a water molecule in the liquid to leave the aqueous phase in favor of a relatively weak interaction with the keto C=O function will not be very strong, whereas a molecule water in the gas phase may well tend to interact with solid chlorophyll.

The hydration behavior of chlorophyll films appears to be very sensitive to the orientation of the chlorophyll molecules. This may be the primary reason why films cast from different solvents interact so differently with water. Studies of the aggregation of chlorophylls in solution have shown[13] that the chlorophyll species present in different solvents vary widely. From a solution in *n*-hexane, large oligomers will be deposited, whereas, from solutions in carbon tetrachloride, the average state of aggregation will be expected to be smaller. These differences in aggregation are reflected in the infrared and show up strongly in the way various films interact with water (P. DAIGNEAU, K. BALLSCHMITER AND J. J. KATZ, unpublished work).

Pheophytin a–water interactions

If the coordination properties of the magnesium atom in the chlorophyll molecule are the determining factor governing self aggregation and ligand interaction, the magnesium-free chlorophyll, pheophytin, should exhibit basically different behavior. The infrared spectra of films of pheophytin *a* and *b* have been reported by SIDOROV AND TERENIN[21]. Pheophytin is said to form hydrates and the pheophytin–water interaction has been investigated by infrared studies by KARYAKIN *et al.*[24] and KARYAKIN AND CHIBISOV[30]. A recent quantitative study of the hydration and dehydration of chlorophylls and pheophytin *a* did not reveal any defined pheophytin hydrates[20]. In the O–H region of the infrared, however, a more or less intense broad absorption band with the maximum at 3250 cm^{-1} can be observed, depending on the experimental conditions used to cast the film. Heating a film of pheophytin *a* to elevated temperature (50–60°) in a high vacuum (about 10^{-3} mm Hg) has little effect on the extent of this O–H absorption. However, codistillation of the water with carbon tetrachloride results in nearly anhydrous pheophytin (Fig. 1E).

The water in the pheophytin film does not undergo hydrogen bonding to the carbonyl functions, since the position and relative intensities of the bands in the C=O region of the infrared are unchanged upon drying of the pheophytin (Fig. 1E). Hydrogen bonding with the oxygen to a N–H group is likewise unlikely, as the position and intensity of the N–H band at 3400 cm^{-1} are practically unaffected by the drying process. Remaining is the possibility of a hydrogen bond of water to the pyrrole nitrogen. This cannot be ruled out, but neither is there evidence for it, except the position of the maximum at 3250 cm^{-1} of the O–H band.

The position of the N–H stretching vibration of pheophytin *a* at 3400 cm^{-1} is not affected whether the molecule is in solution in carbon tetrachloride or in the condensed state. This suggests, as is the case for other dihydroporphyrins[36], that the N–H group within the molecule is not readily accessible. Evidence for this is given by the relatively slow ^2H_2O exchange rate for the N–H group, even in solution. Strong intramolecular hydrogen bonding of the N–H group as for other dihydroporphyrins seems not be the case for pheophytin *a*. The N–H stretching vibration is shifted to lower frequencies about 60 cm^{-1} less than in other dihydroporphyrins, and it appears at the same wave number as the N–H of the pyrrole in the condensed state (3400 cm^{-1}).

Electronic spectra

One of the principal objects of this work is to correlate the absorption spectra in the visible with the various chlorophyll species present in films as deduced primarily by infrared spectroscopy. We have recorded electronic transition spectra for the films investigated by infrared described above. These spectra are shown in Fig. 7. Since we have recorded these data, we have developed deconvolution techniques that contribute substantially to the interpretation of the visible absorption spectra of chlorophyll oligomers[37] but the applications of these methods to films is still not fully worked out. We, therefore, content ourselves at this time with a presentation of the experimental results and some comment on the more unusual features of the electronic spectra. The effects of water on the electronic absorption spectra of films of chlorophyll have been reported by LITVIN AND GULYAEV[38], BYSTROVA AND KRASNOVSKII[39], KRASNOVSKII AND BYSTROVA[40] and SHERMAN AND LINSCHITZ[25].

Particularly to be noted are the complicated nature of the changes in the visible absorption spectra of the chlorophylls on hydration. Where hydration occurs

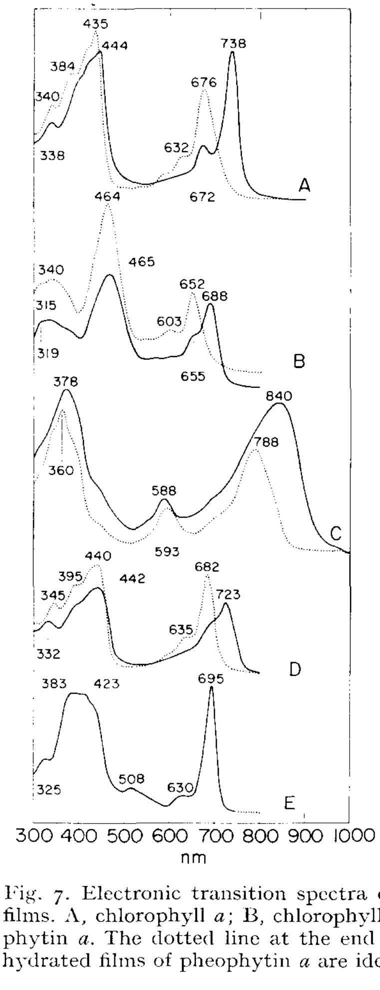


Fig. 7. Electronic transition spectra of anhydrous (———) and hydrated (———) chlorophyll films. A, chlorophyll *a*; B, chlorophyll *b*; C, bacteriochlorophyll; D, pyrochlorophyll *a*; E, pheophytin *a*. The dotted line at the end of Curve E is meant to indicate that the anhydrous and hydrated films of pheophytin *a* are identical.

with no pronounced changes in the relative orientation of the chlorophyll molecules, as in (Chl $a)_2 \cdot H_2O$, (Chl $b)_3 \cdot H_2O$, (Chl $b)_3 \cdot 2H_2O$, then the consequences of hydration on the spectra are minimal, and hydrates and anhydrous films of oligomers have very similar spectra. Where extensive reorientation can be expected, as in $(Chl \cdot H_2O)_n$, $(BChl \cdot H_2O)_n$, or (Chl $b \cdot H_2O)_n$, then very marked red shifts are observed.

It is possible to recognize in a qualitative way that major reorientations of the chlorophyll molecules must occur in the formation of the 1:1 hydrates. For the chlorophyll *a* monohydrate the red shift is about 62 nm (relative to the chlorophyll *a* oligomer film), but for the corresponding chlorophyll *b* monohydrate it is only 36 nm, and for pyrochlorophyll *a* monohydrate, it is 31 nm. This may mean that the distances between chlorophyll molecules, or the angle between them, is greater for pyrochlorophyll *a* and chlorophyll *b* than for chlorophyll *a* itself [41–43]. In the case of bacteriochlorophyll, a large red shift of about 53 nm is observed in hydrated films. The shift observed here is the same as observed for some forms of bacteriochlorophyll *in vivo*, and suggests that in both cases a similar organization of the bacteriochlorophyll molecules involving interaction with water is responsible for the red shift.

Films of pheophytin *a* show a remarkably large red shift (Fig. 7); the red maximum, which appears at 668 nm for pheophytin in hydrocarbon solutions, is found at 695 nm in the films. The spectrum is unchanged when the pheophytin *a* film is exposed to water vapor. This large red shift in anhydrous films of pheophytin may result in a strong plane–plane interaction as observed for other porphyrins[23]. The chlorophylls, however, because of the presence of the central magnesium atom, have much greater difficulty in assuming parallel configurations and thus exhibit much more variable behavior.

It must be emphasized again that the electronic transition spectra are characteristic of particular chlorophyll species, as a whole, and cannot be assigned to individual chlorophyll molecules. It is also becoming increasingly evident that the production of long wavelength forms of chlorophyll requires the interaction of two or more molecules of chlorophyll. Long wavelength forms of chlorophyll result from a cooperative interaction, either by direct interaction of the chlorophyll molecules, or by an interaction mediated by a bifunctional ligand such as water[44].

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