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AGGREGATION OF CHLOROPHYLLS IN MONOLAYERS

V. THE EFFECT OF WATER ON CHLOROPHYLL *a* AND CHLOROPHYLL *b* IN MONO- AND MULTILAYER ARRAYS

Camille CHAPADOS and Roger M. LEBLANC

Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7

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The nature of the interactions between water molecules and monolayers and multilayers of chlorophyll *a* (Chl *a*), and monolayers and multilayers of Chl *b*, obtained by the Langmuir-Blodgett technique, is examined by infrared spectroscopy. Following deposition of the monolayer or multilayer of Chl *a* or Chl *b* onto a plate, repetitive scans showed some modifications in the infrared spectra which are interpreted as a reorganization of the molecules as some water molecules leave the array. Drying the sample further modifies the spectra, which indicates the departure of more tenacious water molecules. Putting the sample in a moist atmosphere does not restore the original spectrum. This is an indication of a nonreversible reorganization in the chlorophyll array. The spectra of the monolayer of chlorophyll are much more complicated than those of the multilayer, owing to the nonintegrating effect of the monolayer, which reveals the perturbing effect of the different dielectric milieux on each functional group. On the basis of the analysis of the spectra and the information gathered from the surface pressure isotherms, a model is proposed for the monolayer arrangement at the air/water interface which implies two set of dimers of water per molecule of chlorophyll. One pair of dimers constitutes the water of the first kind and is composed of vapor-like dimers. This kind of water is situated between the porphyrin planes of chlorophyll molecules and is easily removed from the monolayer. The second pair of dimers is composed of water of the second and third kinds situated between the Mg atom of the chlorophyll molecules and the water of the subphase. The second kind of water is closest to the Mg atom and is the most difficult one to remove. The third kind of water is closest to the surface and its mobility is intermediate between that of water of the first kind and that of water of the second kind. Comparing the infrared spectra of a freshly prepared monolayers of Chl *a* with the resonance Raman spectra of intact chloroplasts (M. Lutz, Biochim. Biophys. Acta 460 (1977) 408), we notice great similarities. This is an indication that the model we propose for the monolayer of Chl *a* could play an important role in the chloroplast.

1. Introduction

Water can participate in the aggregation state of chlorophyll molecules, present in the chloroplast, in many different ways and can play an important role in the photosynthetic process. In this system, in spite of many different studies, there is no definitive model of the association of water with chlorophyll. In the chloroplast, the chlorophyll molecules are oriented and to simulate this arrangement we have been using the monolayer assembly technique to study in part II of this series [1] the interactions of chlorophyll with dioxane using the techniques of infrared and electronic spectroscopies. In part III [2], we have studied by infrared spectroscopy the mono- and multilayer arrangement in Chl *a*. In part IV [3], we have examined the reorganization of Chl *a* in a multilayer array using the same spectroscopic techniques, and in a subsequent paper [4], the effect of pollutant gases on Chl *a* multilayers. In the present paper we report an infrared spectroscopic study of the influence of water on Chl *a* and Chl *b* in mono- and multilayer arrays.

Infrared spectroscopic techniques have previously been used to determine chlorophyll-water interactions in different aggregated states. Katz and Ballschmitter [5–7], have studied chlorophyll-water interactions in various solvents, and the interaction of water on solid films of chlorophyll obtained by evaporation of the solvent [8]. Sherman and Wang [9,10] have studied the interaction of water with Chl *a* suspended in mineral oil. Resonance Raman spectroscopic techniques have been used by Lutz and co-workers to study chlorophyll in solution [11–13], in the solid state [13], and in intact chloroplasts [11,13–15].

Electronic spectroscopy has been employed extensively to study chlorophyll in different associations with water. While this technique can easily distinguish between crystalline and amorphous chlorophyll [8,9], it is not very sensitive to small variations in the aggregation state [3]. The electronic spectra of the effect of water vapor on Chl *a* double layers have revealed [1] that after 90 h of exposure to water vapor, a small band appears at 735 nm while the 680 nm band is decreasing. Leaving the sample in a dry atmosphere does not lead to the reappearance of the original spectrum. The situation is different in a solid film where the hydration of Chl *a* molecules is much more rapid, giving rise to a strong band at 738 nm [8,9]. In the monomolecular array, the orientation of Chl *a* molecules does not favor the 740 nm Chl *a*-water complex.

Monolayer and multilayers of Chl *a* molecules have different aggregation states [2] so that the model of interaction between Chl *a* and water that we have proposed for the Chl *a* multilayer [3] will be different in the monolayer. The aggregation pattern will be different in Chl *b* compared to Chl *a* because of the supplementary carbonyl group. It is the object of this paper to study by infrared spectroscopy the interaction of water with Chl *a* and Chl *b* in a mono- and multilayer organization in order to have a model of aggregation of chlorophyll molecules in a system that has the pigments oriented in a fashion similar to that of the in vivo situation. We hope that this process will help to clarify some of the unanswered questions, recently mentioned by Gaines [16], about the structural aspect of monolayers compared to multilayers.

2. Experimental

The materials, preparation of slides, deposition of monolayers, float trough and Langmuir film balance apparatus have been described previously in detail [17,18]. For multilayers, deposition onto a plate was done by dipping and withdrawing the plate from the trough. For monolayers, deposition was done only when withdrawing the plate.

The extraction and purification of Chl *a* from spinach leaves were performed by the standard method of Strain and Svec [19].

To determine the purity of samples, we have used as before [3] the following criteria: the surface pressure-area isotherm, the absorbance ratio of the blue to the red band, and thin-layer chromatography. Only those samples that had good criteria were used for the experiments.

2.1. Infrared spectra

Layers of Chl *a* or Chl *b* were deposited at a surface pressure of 20 mN m^{-1} on each side of a multiple internal reflexion (MIR) plate using the Langmuir film balance to monitor deposition. For Chl *a*, the multilayer is composed of 40 layers deposited on each side of an AgBr MIR plate; for Chl *b* the multilayer is composed of 41 layers deposited on each side of a germanium MIR plate. For the monolayer of Chl *a* and of Chl *b*, germanium MIR plates were used.

For multilayers, the infrared spectra were taken on a Unicam SP 200G spectrophotometer using an abscissa scale-expansion device in order to increase the precision of the bands. The precision of some spectra was checked with a Perkin-Elmer model 180 spectrophotometer. For monolayers, the latter spectrophotometer was used with an ordinate scale expansion of 10. The precision of the bands is ±1 cm^{-1} and the resolution of the instruments approx. 4 cm^{-1}.

To understand the influence of water on monomolecular arrays of chlorophyll, we took repetitive infrared spectra of the sample after each stage of the procedure. The different stages are: (1) after preparation of the sample of chlorophyll, it is transferred as quickly as possible from the trough to the infrared sample compartment; (2) the sample is dried at 75°C for the multilayer and at 65°C for the monolayer in a vacuum chamber under a vacuum of 0.1 Pa; (3) the sample is put in a closed container with water vapor for a period of time; (4) the sample is dried again in the vacuum chamber.

3. Results and discussion

3.1. Infrared spectra of Chl a

The spectra of the multilayers are presented first because they are less complicated than those of the monolayer, and because they can be compared more easily to other systems.

3.1.1. Multilayer of Chl a

Fig. 1A shows the spectrum of a freshly prepared multilayer taken 25 min after the end of deposition. The assignments of the bands are given in table 1. With time the 1662 cm^{-1} band is displaced to 1665 cm^{-1}, accompanied by a de-

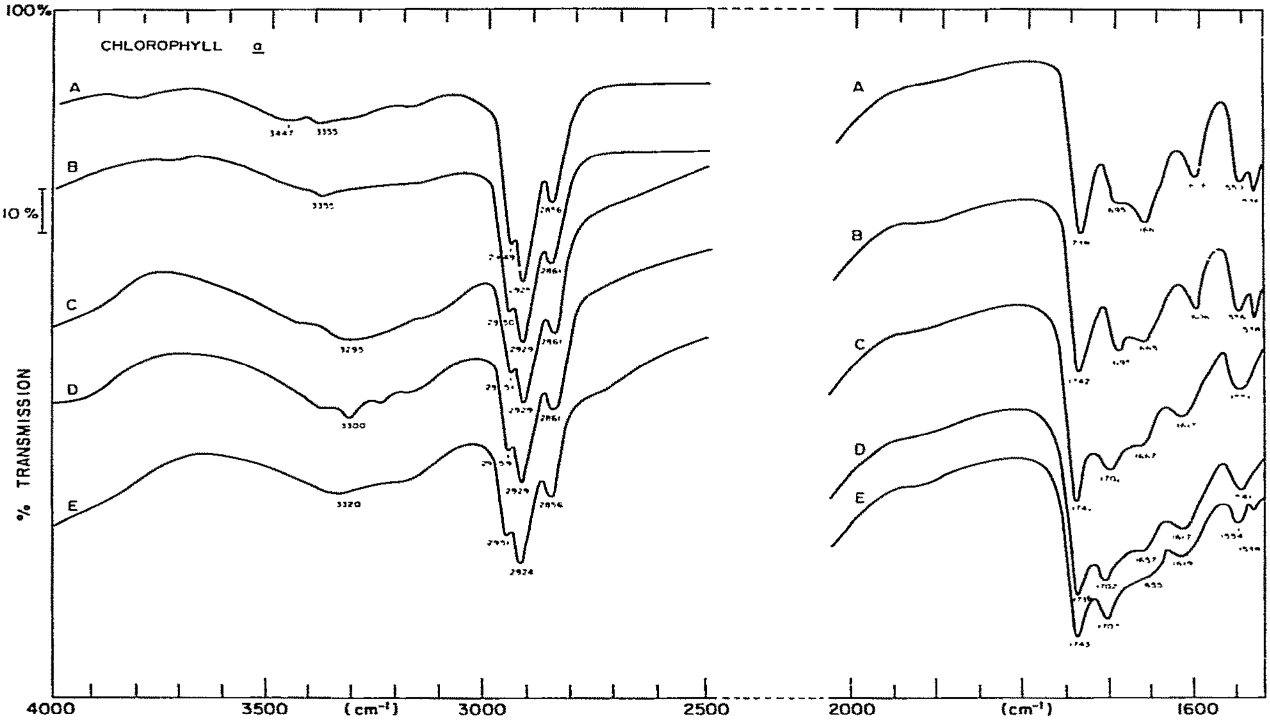


Fig. 1. Infrared spectra of the effect of water vapor on a Chl *a* multilayer (2×40 layers). (A) Spectrum taken 25 min after the end of deposition; (B) multilayer after reorganization and after being dried under vacuum (0.1 Pa) for 60 min at 75°C; (C) multilayer exposed to water vapor (≈ 3.2 kPa) for 150 min at 25°C; (D) multilayer dried for 63 h in a dessicator; (E) multilayer dried under vacuum (0.1 Pa) for 6 h at 75°C.

Table 1

Wave number positions (cm^{-1}) of Chl *a* in the carbonyl region

Spectrum and description			Ester C=O			
		1820–1750	1750–1741	1740–1737	1736–1731	1730–1717
1. Multilayer						
(A)	25 min after deposition			1738		
(B)	After vacuum, 75°C, 1 h		1742			
(C)	Water vapor, 25°C, 2.5 h		1742			
(D)	Dried, 25°C 63 h				1736	
(E)	Dried, 75°C, 6 h		1743			
2. Monolayer						
(B)	15 min after deposition			1739	1735	
(C)	25 min after		1742sh	1737		1728sh
(D)	35 min after			1740		
(E)	71 min after	1795 1777			1735	
(F)	After vacuum, 65°C, 2 h	1760	1742sh	1737		1730
(G)	10 min after			1738		1728
(H)	20 min after			1737		1722
(I)	55 min after	1768	1746sh		1735	
(J)	105 min after	1760		1740	1736	
(K)	115 min after	1758sh		1737		
(L)	125 min after		1742		1734	1730
(M)	After 10 h, water vapor, 25°C			1738		1723
(N)	10 min after	1762		1737		1719
(O)	20 min after				1735	
(P)	30 min after	1784 1770	1744sh		1736	
(Q)	75 min after	1800 1766			1736	
(R)	145 min after	1778		1740		1730
(S)	After vacuum, 25°C, 1.5 h	1765		1740sh	1735	
(T)	10 min after			1737	1731	
(U)	20 min after				1735	1722
(V)	30 min after	1760		1740		1727
(W)	After vacuum, 65°C, 1.5 h	1762	1743	1737		1727
(X)	10 min after	1760	1744		1734	1729, 1720
(Y)	20 min after	1765	1744		1734	
(Z)	30 min after	1820, 1801 1779, 1754	1742			1730
(ZZ)	80 min after	1811, 1762		1737		1720
(ZZZ)	220 min after	1821, 1805 1782, 1768			1736	
Mean ± S.D.			1743 ± 1	1738 ± 1	1735 ± 1	1726 ± 4

Table 1 (continued)

Free keto C=O					Associated keto C=O			
1716– 1705	1704– 1700	1699– 1694	1693– 1686	1685– 1676	1675– 1668	1667– 1660	1659– 1648	1647– 1630
		1695				1662		
		1695				1665		
	1702					1667		
	1702						1657	
1707							1655sh	
1714	≈ 1703sh	1697	1687sh	1676			1653	≈ 1640
1708sh		1697	1690sh			1663		
1716sh	1700			1682	1668		1656	1638
		1697		1676		1666		1635
		1698		1682		1665		
1709sh	1702	1697	1690sh			1665	1656	1638
		1697	1690	1677			1657	1644
		1697				1664		
1715		1697	1686			1663		1644
		1697						
	1703	1695				1666		
		1697				1665		1640
	1702	1696			1675			1638
	1701sh	1697	1690		1672		1654	≈ 1640
		1699		1680		1666	1648	1634
1714		1698		1678			1658	1638
		1696						1631
		1698	1690sh	1680sh			1653	
		1698		1682		1660		1640
		1698				1666		1638
	1702	1697		1680				1640
1714	1700				1672		1654	1641
	1701	1696	1690sh	1676			1655	1639
	1701	1696					1650	
1710		1697		1682		1662		1640
								1631
		1695						
		1697						1642
1712 ± 3	1702 ± 1	1697 ± 1	1689 ± 2	1679 ± 3	1672 ± 3	1664 ± 2	1654 ± 3	1638 ± 4

Table 1 (continued)

Spectrum and description	Skeletal C=C, C=N							
	1630–1617	1615–1606	1606–1585	1584–1561	1560–1541	1540–1520	1520–1510	1509–1499
1. Multilayer								
(A) 25 min after deposition		1606			1550	1536		
(B) After vacuum 75°C, 1 h		1606			1556	1538		
(C) Water vapor, 25°C, 2.5 h	1617				1551			
(D) Dried, 25°C, 63 h	1617				1541			
(E) Dried, 75°C, 6 h	1619				1554	1538		
2. Monolayer								
(B) 15 min after deposition		1609						
(C) 25 min after		1608						
(D) 35 min after	1620	1607						
(E) 71 min after	1625	1608	1593	1580	1553 1544	1535 1531	1512	1501
(F) After vacuum, 65°C, 2 h	≈ 1630	1616						
(G) 10 min after	1619	1608						
(H) 20 min after	1621							
(I) 55 min after	1620			1574	1550	1526	1510	
(J) 105 min after	1628	1613						
(K) 115 min after	1617							
(L) 125 min after	1622	1612						
(M) After 10 h, water vapor, 25°C		1615	1605					
(N) 10 min after	1621	1611						
(O) 20 min after	1624	1609						
(P) 30 min after	1618			1578				
(Q) 75 min after	1623	1613	1600	1576				1508
(R) 145 min after				1582	1551			1499
(S) After vacuum, 25°C, 1.5 h	1625		1603					
(T) 10 min after	1625	1608						
(U) 20 min after	1620							
(V) 30 min after					1545	1525	1515	
(W) After vacuum, 65°C, 1.5 h	1630	1616	1604					
(X) 10 min after		1610	1603					
(Y) 20 min after	1618							
(Z) 30 min after	1622	1605	1596	1579	1552	≈ 1530	≈ 1512	
(ZZ) 80 min after	1618			1580	1552	1541	1516	
(ZZZ) 220 min after	≈ 1625	≈ 1610		1578	1550	1534	1518	1508
Mean ± S.D.	1622 ± 4	1610 ± 3	1600 ± 4	1578 ± 3	1550 ± 4	1533 ± 5	1514 ± 3	1504 ± 5

crease in intensity and a comparative increase in the 1695 cm^{-1} band. After stabilization and after being dried under vacuum (0.1 Pa) for 60 min at 75°C, spectrum B is obtained. After the multilayer is exposed to water vapor at 25°C for 150 min, spectrum C is obtained where the 1695 cm^{-1} band is displaced to 1702 cm^{-1}, the intensity of the 1665 cm^{-1} band is further decreased, the 1606 band is displaced to 1617 cm^{-1} and its intensity pattern modified, and the doublet at 1556–1538 cm^{-1} is modified to give one band at 1551 cm^{-1}. When the multilayer is dried in a desiccator for 63 h, spectrum D is obtained; in the carbonyl region there are some small displacements of the bands and some slight modifications of the intensity pattern. Further dehydration, by heating the multilayer at 75°C under vacuum (0.1 Pa) for 6 h, will restore the doublet at 1554–1538 cm^{-1} but will further decrease the band at 1655 cm^{-1}. In the C-H region at approx. 2925 cm^{-1}, there are some small displacements of the bands with the various treatments that the multilayer undergoes but with no drastic modifications. In the O-H region, the small bands at approx. 3400 cm^{-1} of the freshly prepared multilayer (spectrum A) are hardly modified by the effect of time or heat treatment (spectrum B). These small bands are not always present (see ref. 3) and when they are present they cannot be unambiguously assigned to water [4]. Upon exposure to water vapor (fig. 1C) the band is increased and is displaced to 3300 cm^{-1}. After being dried in a desiccator (fig. 1D) the band is decreased. Drying the multilayer at 75°C under vacuum for 6 h decreases further the intensity of the band (fig. 1E).

To understand the influence of water molecules on the Chl *a* multilayer, we have to refer to the model of aggregation of Chl *a* in multilayer arrays that was proposed in part IV [3]. In summary, the model we have proposed for a freshly prepared multilayer consists of a polymer of Chl *a* molecules linked together by the carbonyl ketone group of one molecule with the Mg atom of the other molecule through a C=O---Mg coordinate bond, and held together by two molecules of water, hydrogen bonded together, and linked to one molecule of Chl *a* by an Mg---O coordinate bond and to the other molecule of Chl *a* by an OH---π hydrogen bond. With a freshly prepared multilayer, the number of polymeric links is greatest so that the free ketone band at approx. 1695 cm^{-1} would be absent immediately after the multilayers have been prepared. As the more labile molecule of water leaves the multilayer aggregation, the multilayer collapses, breaking many polymeric links as evidenced by the decrease in the ketone C=O---Mg band at 1662 cm^{-1} (fig. 1A and B) to the benefit of the free ketone band. Heating the sample (fig. 1B) at 75°C under a vacuum of 0.1 Pa for 1 h should not substantially eliminate the more stable water molecule linked to the Mg atom of the Chl *a* molecule because only drastic means, like the influence of SO_2 molecules or a high vacuum (0.1 mPa), can abstract the water molecule from the Chl *a* environment. The reorganization of a freshly prepared multilayer of Chl *a* was fully treated in ref. 3.

The influence of water vapor on a dry multilayer of Chl *a* gives rise to a structureless band situated at 3300 cm^{-1} (fig. 1C) and it perturbs the skeletal C=C, C=N vibrational bands as shown by the fusion of the skeletal doublet at 1556–1538 cm^{-1} to give one band at 1551 cm^{-1} and by the modification of the 1606 cm^{-1} band. The water molecules must form some hydrogen bonds with the π-electron network of the Chl *a* multilayer. These water molecules perturb the multilayer array, causing some further rupture of the remaining ketone carbonyl C=O---Mg coordinate bond as shown by the decrease in the 1667 cm^{-1} band. When the multilayer is dried in a desiccator for 63 h, the OH band is hardly affected by the process, the skeletal bands are displaced a little, so is the coordinate ketone band and its intensity is slightly increased. With more drying under vacuum at 75°C (fig. 1E) the O-H band is further decreased, the skeletal doublet is restored, but the coordinate ketone band approx. at 1655 cm^{-1} is decreased. This can be explained by the shuffling of the multilayer when water molecules leave the Chl *a* environment, causing some further rupture of the C=O---Mg coordinate bonds. This phenomenon has been previously observed [3].

On a solid film of Chl *a* [8], the influence of water vapor is to give in the infrared region a strong band situated at 1640 cm^{-1} for the associ-

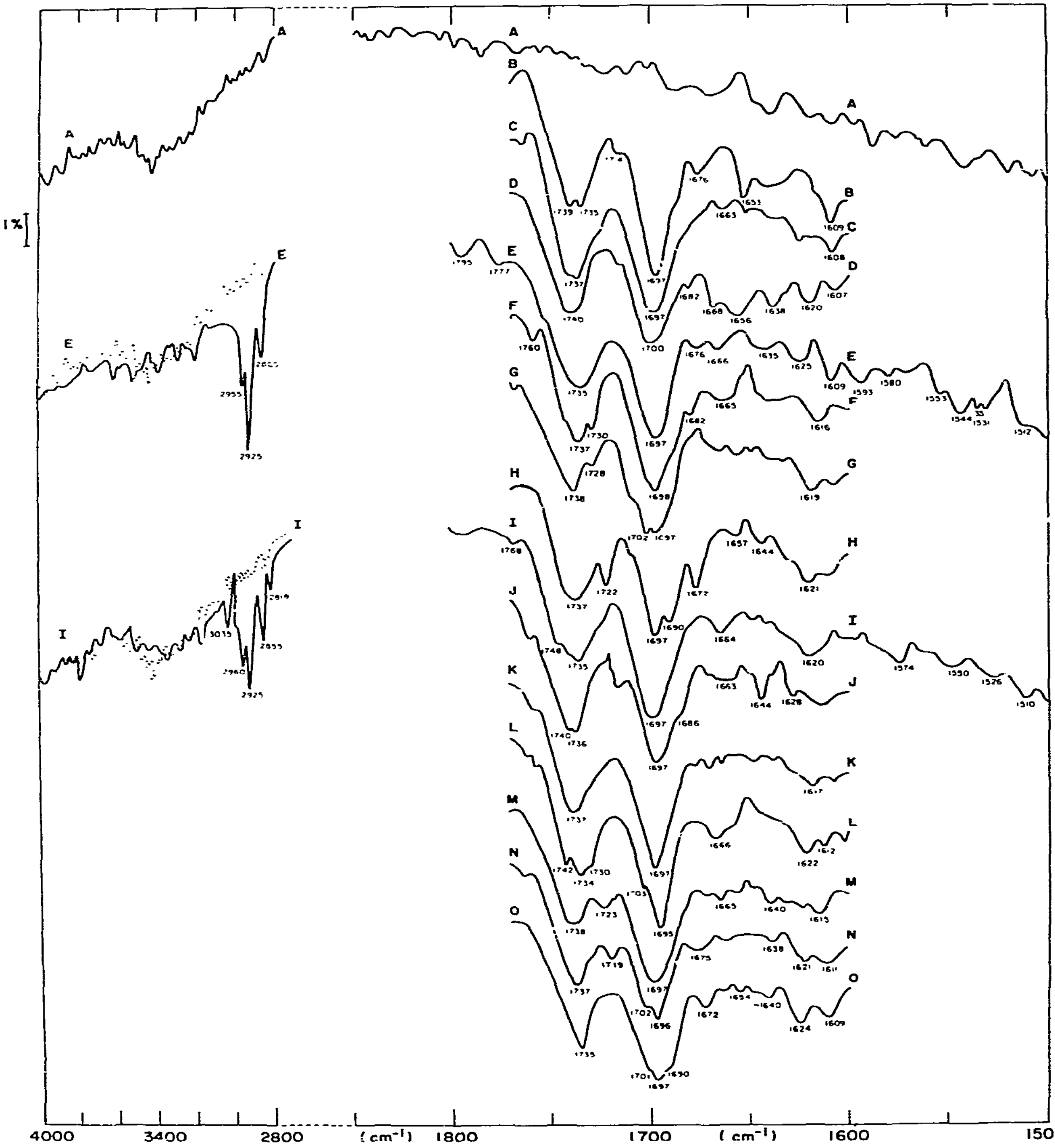
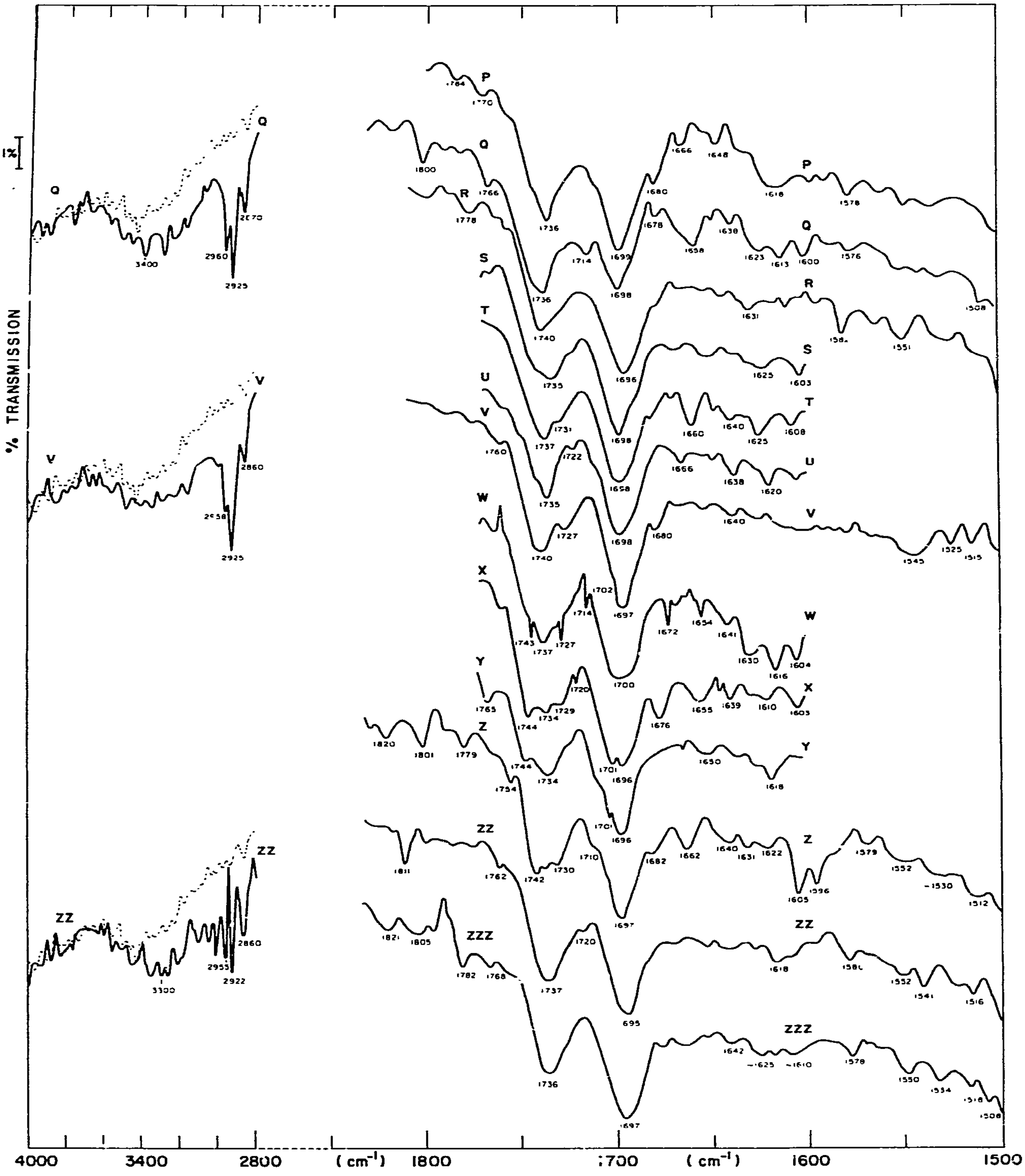


Fig. 2. Infrared spectra of the effect of water vapor on a Chl *a* monolayer (2×1 layer). (A) Background; (B) sample, 15 min after deposition; (C) 25 min after; (D) 35 min after; (E) 70 min after; (F) after heating at 65°C under vacuum (0.1 Pa) for 2 h and after cooling down; (G) 10 min after; (H) 20 min after; (I) 55 min after; (J) 105 min after; (K) 115 min after; (L) 125 min after; (M) after 10 h in water vapor at 25°C (= 3.2 kPa); (N) 10 min after; (O) 20 min after; (P) 30 min after; (Q) 75 min after; (R) 145 min after; (S) after sample was under vacuum (0.1 Pa) for 1.5 h at 25°C; (T) 10 min after; (U) 20 min after; (V) 30 min after; (W) after sample was under vacuum (0.1 Pa) for 1.5 h at 65°C; (X) 10 min after; (Y) 20 min after; (Z) 30 min after; (ZZ) 80 min after; (ZZZ) 220 min after. The dashed line is spectrum A reproduced.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ated ketone carbonyl and a weaker band situated at 1695 cm^{-1} for the free ketone carbonyl. Apart from a small shoulder appearing on the lower side of the skeletal doublet, there are not very many modifications of the C=C, C=N bands. The 1640 cm^{-1} band has been assigned [8] to a ketone C=O- - -H-O-H- - -Mg organization forming a polymer of hydrated Chl *a* molecules.

The hydrated dry multilayer * of Chl *a* gives an infrared spectrum which is different from that of a hydrated solid film, or that of a freshly prepared multilayer. The aggregation state of the hydrated dry multilayer must therefore be different for these two aggregation states.

3.1.2. Monolayer of Chl a

The spectrum of a freshly prepared monolayer of Chl *a* on a Ge MIR plate 15 min after the end of deposition is given in fig. 2B followed by spectra C–E to monitor the effect of time. After spectrum E, the following treatments have been carried out followed by repetitive scans: drying at 65°C under a vacuum of 0.1 Pa for 2 h (fig. 2F–L); being placed in contact with water vapor at 25°C for 2 h (fig. 2N–R): drying at 25°C under a vacuum of 0.1 Pa for 1.5 h (fig. 2N–V): and drying at 65°C under a vacuum of 0.1 Pa for 1.5 h (fig. 2W–ZZZ). Spectrum A is the background spectrum, in the region 4000–2500 cm^{-1}, which is represented by broken lines in the spectra of the monolayer so as to monitor properly the O-H band.

At first glance, one could be amazed by the several species observed, contrary to what has been observed for a multilayer or for a solid film, and by the many variations in the intensities of the bands with time. One would be tempted to attribute this diversity to instrumental factors, but when small features are reproduced with minor modifications from one spectrum to another and when these features are repeated from one sample to another, one has to reconsider the situration for the monolayer. The assignments of the bands which are fairly certain are given in table 1 for the carbonyl region. The major divisions are as before (refs. 1–3 and fig. 1): the ester carbonyl region, the associated ketone carbonyl region, and the skeletal C=C, C=N region. The subdivisions in the major divisions are made in order to bring together the bands of the same vibrational origin in the same apparent dielectric milieu.

The first column covering the region 1820–1750 cm^{-1} presents some values of small bands which show some constancy. These bands cannot be identified unambiguously because they are just beyond the region of the ester carbonyl. They could originate from a combination or harmonic of some lower fundamentals that an aggregation state would render more visible. They could also originate from some carbonyls of transient species.

The ester carbonyl range covers the region 1750–1717 cm^{-1}, the highest value being a shoulder at 1746 cm^{-1} (fig. 2I) and the lowest at 1722 cm^{-1} (fig. 2U). Using Buckingham's equation [20] that employs the dielectric constant (ϵ) and refractive index (n) for the evaluation of solvent effects in infrared spectroscopy, Bekarek et al. [21] have determined that the position of the ester carbonyl band in the state of an ideal gas is 1740 ± 2 cm^{-1} which confirms the mean position at 1743 ± 1 cm^{-1} that we obtain for the bands in the 1750–1741 region (table 1). Although the state of an ideal gas for such a bulky molecule as chlorophyll in the solid state may be considered improbable, it is nonetheless not impossible that a few molecules or at least a few functional groups behave spectroscopically in the same way as if they were in an ideal gas situation. In the cases observed, the bands at approx. 1743 cm^{-1} appear as shoulders or small bands on the main band, indicating that only a few molecules behave in this manner.

The bands situated in the 1740–1737 and 1736–1731 cm^{-1} regions constitute the bulk of the absorption of the ester carbonyls; it is divided into two regions because in some instances we clearly see a doublet as shown in fig. 2B, an indication of different species. The 1730–1717 cm^{-1} range covers the lower frequency region of the ester carbonyl band where absorption appears as satellite components.

The free ketone carbonyl range covers the re-

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* A hydrated dry multilayer is a dry multilayer which has been hydrated.

gion 1716–1676 cm^{-1}. By free ketone we mean free from the interaction with Mg atoms of neighboring molecules and with H_2O molecules. The value calculated by Bekarek et al. [21] of 1711 ± 2 cm^{-1} for the ketone band in the state of an ideal gas is consistent with the mean value of 1712 ± 3 cm^{-1} observed in the 1716–1705 cm^{-1} region. As before, the bands in this region appear as shoulders or weak satellite bands on the main band, an indication of the few molecules in this ideal state. The bulk of the free ketone band is situated in the 1704–1695 cm^{-1} region divided into two because, here again, we observe in some instances the presence (cf. Fig. 2G) of doublets. Weaker bands appear in the 1694–1686 and 1685–1676 cm^{-1} regions.

Chl *a* molecules in different solvents will show an absorption varying from 1741 to 1726 cm^{-1} and from 1704 to 1684 cm^{-1} for the ester carbonyl and the free ketone carbonyl [21]. For a given solvent, there is only one band for each functional group whereas in the monolayer situation we observe several bands whose intensities are slightly modified from one spectrum to another when one scan is followed by another (cf. fig. 2B–E). On one spectrum the different bands can be explained by the different dielectric milieux surrounding the different functional groups. As we will see later, the monolayer is in a dynamic situation. Because of this, the dielectric medium will vary continually, modifying the environment of the functional groups, and therefore their spectra.

The associated ketone carbonyl region covers the region 1675–1630 cm^{-1}. With a monolayer, there is no coordinate CO---Mg bond possible as in the multilayer situation [2], but the presence of clearly identifiable bands, although weak and varying from one spectrum to another, cannot be denied. Since this region is too small to be due to a solvent effect on the free carbonyl ketone, we have to consider other possibilities of interactions. One of these possibilities is the influence of water molecules, another is the interactions with the MIR plate surface. These possibilities will be discussed later.

The skeletal C=C, C=N stretch vibrations are situated in the 1630–1499 cm^{-1} region, divided first into the 1630–1561 and 1560–1499 cm^{-1} subregions, and these subregions are further divided so as to reflect the same entities. These divisions are based on the regions more clearly defined in the multilayer (fig. 1). At first glance, one may be surprised that the skeletal C=C, C=N bands are not more clearly defined in the monolayer arrangement, while they are clearly defined in the multilayer one. In the latter case, we have noted previously that these bands are greatly influenced by environmental factors, but the bands are of about the same intensity as that of the ester band or the free ketone band whereas in the monolayer situation they are much less intense and less localized. The C=C, C=N bands also support the π-electron network, and perturbations in this network caused by movements of the porphyrin ring or by a varying dielectric milieu will also modify the shape of the bands which will result in the formation of many components. In contrast, in the multilayer, the closeness of each molecule will reinforce the π-electron network so that the dielectric milieu will be the same for each C=C, C=N group. This phenomenon will favor the localization of the absorption in narrow frequency ranges and thus will increase the intensity of the bands.

In order to understand the influence of water vapor on a monolayer of Chl *a*, we have to consider a model of aggregation for a freshly prepared monolayer on a Ge MIR plate. A first glance at the background spectrum (fig. 2A) reveals that there is no band in the C-H stretch region even with an ordinate scale magnification of 10, but there is a band near 3400 cm^{-1} that we assign to an OH stretch vibration. Therefore, on the surface of the Ge plate, there are some OH groups that do not depart under the vacuum of 0.1 Pa of a plasma cleaner. These OH groups are consequently linked to the Ge molecules through covalent bonds making the plate hydrophilic. Since the 3400 cm^{-1} band is large, structureless and comparable to the absorption of liquid water, the OH groups are linked together through hydrogen bonds in a fashion similar to that of liquid water; there is no free OH group because the absorption near 3700 cm^{-1} is clear. A side view of the organization of these OH groups is presented in fig. 3A.

At the surface of the water, water molecules are linked together by hydrogen bonds, but the water molecules are more labile than the hydroxyls on the surface of the plate so that the hydrogen bond network is continually changing. The chlorophyll molecules at the air/water interface are linked to the water molecules by weak hydrogen bonds between the carbonyls of the chlorophyll with the hydrogens of the water in a situation similar to that presented in fig. 3B. The hydrogens are continually moving from one association to the other, and this is one of the reasons for the deposition of the monolayer of Chl *a* at the surface of the plate where the hydroxyls are more stable.

The departure of water molecules from the surface of the water constitutes the vapor pressure of water. In the vapor phase, dimers exist with a structure that displays a linear hydrogen bond [22]. Some of these dimers will be trapped in the monolayer and will play an essential role in the monolayer organization. Also, in part IV [3], we saw that the dimers constitute the cementing link between two Chl *a* molecules in a multilayer array and it is therefore worthwhile to look at the structure of this dimer.

From recent molecular beam experiments, Dyke et al. [23] have proposed for the water dimer in the vapor phase the structure given in fig. 4A. The O- - -O distance in the gas phase is 2.98 Å which is greater than in ice, 2.74 Å. The azimuthal angle, θ_a, at the minimum of the potential energy curve is 58°. Varying this angle from -40 to 80° will not increase significantly the energy [22,23] provided the donor hydrogen remains on axis. This model is fully supported by high-accuracy quantum-mechanical calculations [22,23]. The quantum-mechanical studies also support the idea of a cooperative effort taking place in the hydrogen-bonded water. When one hydrogen bond is formed as shown in fig. 4A, the charge distribution within the monomers accentuates the formation of other bonds in a chain-like arrangement. This characteristic is important in the monolayer arrays because the water dimers are trapped in between the porphyrin planes in one case, and between the Mg atoms and the water surface in the other case. Therefore, any mechanism that reinforces bonding in the arrays will be favored.

In the water dimer, when the azimuthal angle $\theta_a = 0$, the overall length of the configuration is increased such that links between the chlorophyll molecules are possible. When sandwiched in between the Chl *a* molecules, the arrangement of the water dimer would be in this open configuration. Such a configuration is presented in fig. 4B. On the left side, the oxygen is bonded to the Mg atom, and on the right side the hydrogens are hydrogen bonded to the π-electrons of the porphyrin. In the case of Chl *b*, as we will see later, one of the hydrogens is linked to the oxygen of the aldehyde carbonyl.

We will now look at the pressure-area isotherms of Chl *a* to determine the geometrical parameters between the Chl *a* molecules and between Chl *a* and the surface of the subphase. The surface pressure-area isotherms of pure Chl *a*, at the nitrogen/water interface taken at 20.0°C, are shown in fig. 5. The deposition was done at a pressure, π, of 20 mN m^{-1}. At this pressure, the area covered by one molecule of Chl *a* is 91.8 Å^2 ($A_{\pi=20} = 91.8$ Å^2 molecule^{-1}). From the X-ray work of Kratky and Dunitz (fig. 6 and ref. 24), we obtain for the cross-section of the porphyrin plane of the Chl *a* molecule a value of 10.22 Å for the perpendicular distance between C_{21} and C_{26} to which we add a value of 2×1.07 Å for the C-H distances and 2×0.30 Å for the covalent radii of hydrogen. The section on the *Z*-axis is therefore 12.96 Å. The perpendicular distance between C_{23} and O_2 is $5.93 + 5.42 + 0.71 = 12.06$ Å (± 0.3 Å depending on the inclination of the carbonyl esters) to which we add a value of 1.07 Å for one C-H distance, 0.30 Å for one covalent radius of hydrogen, and 1.9 Å for one hydrogen bond. The section on the *Y*-axis is 15.33 Å and the surface of the porphyrin plane above the surface of water is 198.7 Å^2. At a surface pressure of 20 mN m^{-1}, the angle of tilt of the porphyrin plane is 62.5° and the distance between the molecules is 7.1 Å. With these parameters, we calculate a distance between the Mg atom of one molecule and the N_1-N_2 of the other molecule to be 7.0 Å (see fig. 7). Taking into account the precision of these values, this distance is the same as that we calculated for the water dimer in the open configuration sandwiched between two Chl *a* molecules (fig. 4B), therefore the water dimer will be situated at this most favored position. Since the overall distance linking the water dimer to the Chl *a* molecule is the same as

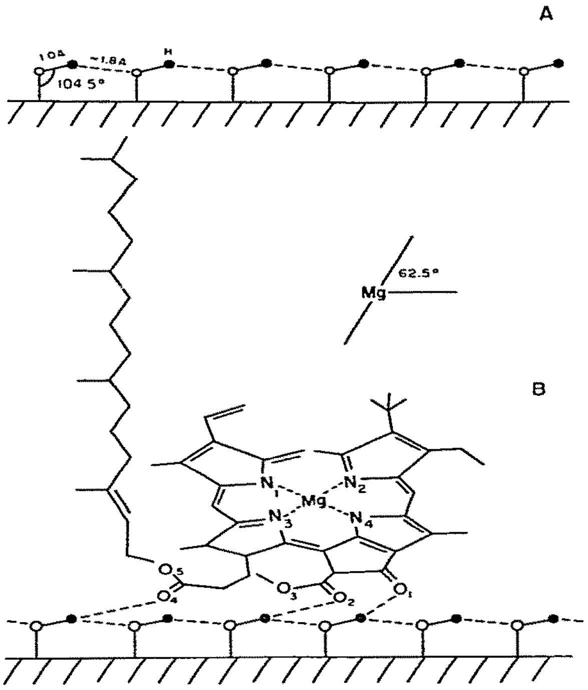


Fig. 3. Model of aggregation of a freshly prepared monolayer of Chl *a* on a hydrophilic MIR Ge plate. (A) Ge MIR plate before deposition; (B) plate with one molecule of Chl *a* (face view without the water molecules).

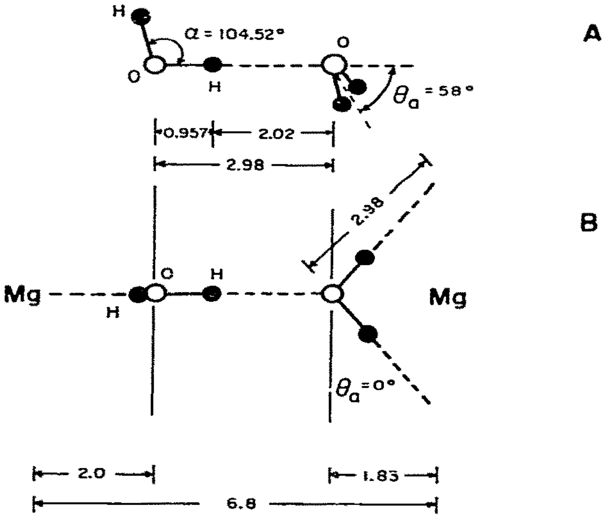


Fig. 4. Structure of the water dimer. (A) Optimal structure in the gas phase according to molecular beam resonance studies [23]; (B) structure in the monolayer array of Chl *a* (top view with $\theta_A = 0°$). Distances are in Å.

for the dimer in the gas phase, the strength of the hydrogen bonds and the coordinate bond will be the same as that of the hydrogen bond of the dimer in the gas phase, and therefore much weaker than in ice. These water molecules constitute the water of the first kind.

The angle of tilt of the porphyrin plane at the water surface is 62.5° at a surface pressure of 20 mN m^{-1}. The distance between the central Mg atom and the surface of the water is 6.3 Å (fig. 7A), a distance sufficient to place two water molecules with shorter intermolecular separations and therefore with stronger bonds than with water of the first kind. The stronger bonds are favored by the position of the Mg atom which is situated 0.43 Å out of the porphyrin plane and by the cooperative effort of the other molecules.

A schematic organization of the Chl *a* molecules at the air/water interface is given in fig. 7. In between the porphyrin planes, there are two water molecules held together by a hydrogen bond, and on one side by a weak coordinate bond with the Mg atom of one Chl *a* molecule and on the other side by weak hydrogen bonds with the π-electron network of another Chl *a* molecule. Because the interactions of these water molecules with the chlorophyll molecules are weak, these molecules constitute water of the first kind and are the ones most likely to depart under a competing situation. This type of water stabilizes the monolayer. Between the surface of the water and the central Mg atom, on the other side of the porphyrin plane, there are two other water molecules linked together by a hydrogen bond. One of these water molecules is coordinate bonded to the Mg atom and constitutes water of the second kind. This water molecule is held firmly in place and can be removed only by drastic means. The other water molecule is hydrogen bonded to the water of the trough and constitutes water of the third kind. The bonding of this type of water with the other molecules is stronger than those of water of the first kind but weaker than the coordinate bond of water of the second kind.

When the Ge plate is pulled out of the trough, one layer of Chl *a* with the accompanying water molecules is transferred onto the plate and the situation at time zero is the same as that at the air/water interface. The carbonyls are linked to the surface hydroxyls by hydrogen bonds with the hydroxyls at the surface of the plate. Since the ester carbonyls are more labile than the ketone carbonyl they must displace themselves in order that the oxygens of the chlorophyll molecule make all the possible hydrogen bonds with the Ge hydroxyl groups. On the Ge plate the hydroxyl groups are linked together by hydrogen bonds and the bonding of these groups with the chlorophyll carbonyls will be weak. This is shown by the fact that the intensity of the associated ketone carbonyls (1675–1630 cm^{-1}) is weak (fig. 2).

After the plate is pulled out, the more labile water in the monolayer organization will depart, destabilizing the organization and modifying the infrared spectra. We will now examine these spectra.

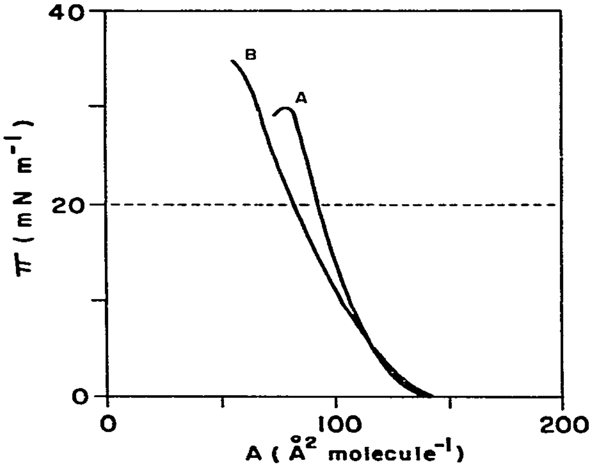


Fig. 5. Surface pressure-area isotherms of pure Chl *a* (A), and pure Chl *b* (B), taken at 20.0°C. Buffer: phosphate, 10^{-3} M, pH 8.0. Spreading solvent: benzene. Deposition is done at a surface pressure, π, of 20 mN m^{-1}.

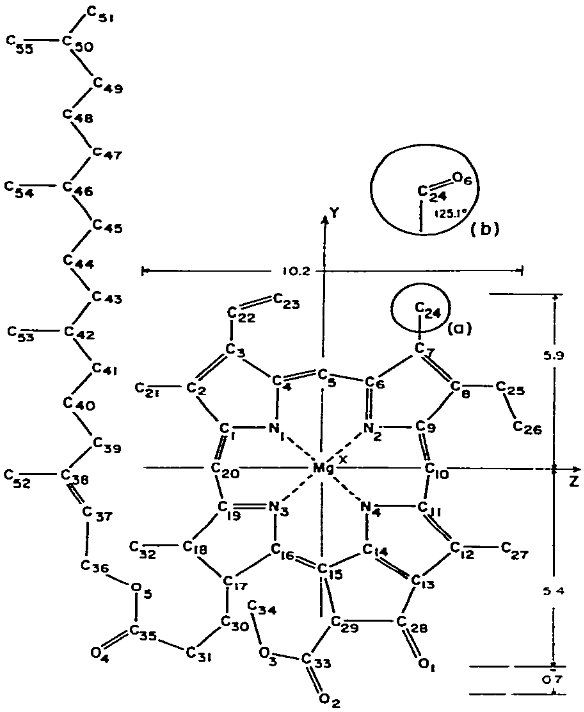


Fig. 6. Structure of chlorophyll based on the X-ray work of Kratky and Dunitz [24]. (a) Chl *a*, (b) Chl *b*. Distances in Å.

3.1.3. Fresh monolayer of Chl a

Looking at fig. 2B, we see the spectrum of a monolayer of Chl *a* taken 15 min after the end of deposition. This spectrum is followed by spectra C–E, taken at time intervals after spectrum B. The Ge plate with one layer of Chl *a* on each side is placed in the dry compartment of the spectrometer.

In spectrum B of fig. 2, we observe for the ester carbonyl a doublet, and with time weaker bands will appear (spectra C and D). All these bands will fuse into one large component (spectrum E). These modifications are indicative of a variety of transient species.

The ketone carbonyl region is situated between 1716 and 1630 cm^{-1}. It is divided into free ketone carbonyl and associated ketone carbonyl (table 1). The former is the more abundant, being composed of at least five discernible species of which the

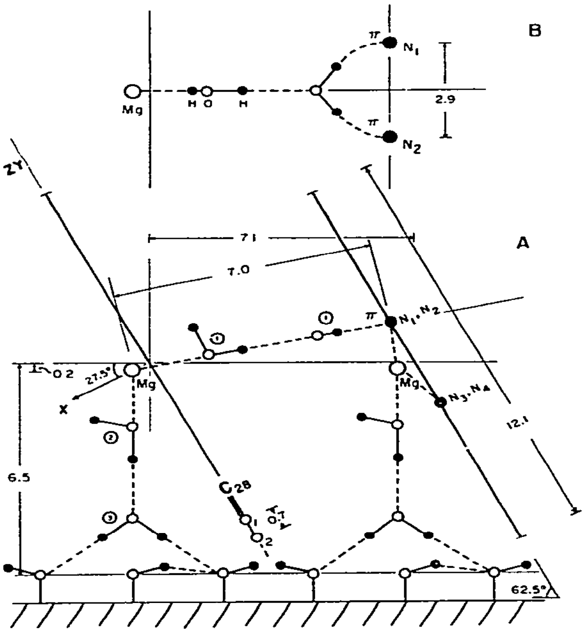


Fig. 7. (A) Schematic representation of the Chl *a* molecules at the air/water interface incorporating the water molecules. Side view. The labels 1, 2, and 3 indicate water of the first, second, and third kind (O_1 and O_2 are hydrogen bonded to some hydrogen atoms that are not shown). (B) Top view of the Mg N_1 N_2 plane. Distances are in Å; angle of tilt is 62.5° at $\pi = 20$ mN m^{-1}.

1697 cm^{-1} component is the most abundant species. The associated ketone carbonyl is composed of at least four discernible species whose intensities vary in time in an apparently erratic manner. However, the integrated intensity of these components is inversely proportional to the intensity of the 1697 cm^{-1} component. Since the association of the ketone carbonyl with the Mg atom of the adjacent chlorophyll molecule is forbidden because of steric considerations, the only possibility of association of this group is with the surface hydroxyl or with water molecules. The latter could come from water of the third kind when it strays from its original organization. If this were the case, it would suppose a great lability of the chlorophyll molecules in order to accommodate the water molecule between the carbonyl and the surface hydroxyl. Because of the many components in the associated ketone region and their modifications with time, it is difficult to present a complete model of the organization. A possible scenario would be the following: in spectrum B, some of the ketone carbonyls are associated with the surface hydroxyl; the 1653 cm^{-1} band would be an indication of this association. In spectrum C, this band has almost disappeared, indicating the breaking of this bond. In spectrum D, the medium-intensity band at 1656 cm^{-1} would indicate that a new bond has been formed either with the surface hydroxyl again, or with water. In spectrum E, this band has almost disappeared, indicating that this bond is ruptured again. This scenario is concerned with the most intense components in the associated ketone region and does not account for the less intense features that are present and which are indicative of the presence of other transient species of lesser importance.

The skeletal C=C, C=N region (1630–1500 cm^{-1}) contains many components (table 1) whose intensity varies with time, and with hydration conditions of the monolayer. In general, the intensity of the bands in this region is less intense than that for the multilayer, solid film or solution. In spectrum B (fig. 2) one band is situated at 1609 cm^{-1}, a position which is comparable to what has been given for the multilayer (fig. 3 in ref. 3) but with a decreased intensity. 10 min later (fig. 2C), the intensity of this band is decreased and a new small band has appeared at approx. 1630 cm^{-1}. Later on (spectrum D), the intensity of the 1608 cm^{-1} component has still decreased and the 1630 cm^{-1} component has given rise to a well resolvable band at 1620 cm^{-1} which is displaced to 1625 cm^{-1} in spectrum E and slightly decreased in intensity while the 1608 cm^{-1} component has slightly increased in intensity. In this spectrum, we see also the 1550 cm^{-1} region where we can indentify four discernable components and in the 1500 cm^{-1} region, two more. In a multilayer (fig. 1), we usually find two components and sometimes three but no more [3]. The many varying components of the skeletal C=C, C=N vibrations of the monolayer are an indication of the many modifications it goes through with time in a dry compartment of an infrared spectrophotometer. These modifications arise with the departure of water molecules of the third kind which would affect the coordination properties of the Mg atom, and the π-electron network. This would influence the C=C, C=N vibrations.

In the O-H region (fig. 2E), there is a small band situated slightly above 3500 cm^{-1} with some apparent features. We assign this band to water molecules in the monolayer. Below 3000 cm^{-1} we see the C-H stretch vibration bands that will not be fully discussed here except to mention that there are some modifications of these bands with aggregation state, indicating movements of these groups during the perturbation.

In the chloroplast, chlorophyll molecules can associate with several molecules present: lipids, protein and water. It is impossible to study intact chloroplast by infrared spectroscopy, however, this can be done by resonance Raman spectroscopy. Lutz [13] has done so in a study of antenna Chl *a* and Chl *b* from chloroplasts of green plants and from monocellular algae. From *Botrydiopsis alpina*, a monocellular alga devoid of Chl *b*, Lutz obtained the components given in table 2 in the carbonyl region at 35 K. One more component situated at 1614 cm^{-1} is given. It is assigned to a skeletal vibration, and although the 1638 cm^{-1} component is not assigned, all the other components are assigned to the C=O ketone vibrations. There is no component mentioned at 1698 cm^{-1} but on the spectrum we see a shoulder comparable to the 1691 cm^{-1} component. The ester carbonyls are not seen by this technique. Resonance Raman spectroscopy of intact chloroplast of several other species gave similar results. Comparing the resonance Raman spectra of intact chloroplast with those of Chl *a* and of Chl *b* in monomeric, oligomeric and hydrated polymeric states, Lutz [13] has proposed that, for antenna chlorophyll molecules, the bonding is with foreign molecules, probably protein through hydrogen bonds with the ketone carbonyl and coordinate bonds with the Mg atom of the chlorophyll molecules.

While the intensity of the bands observed by resonance Raman spectroscopy at low temperature is not directly comparable to those observed by infrared spectroscopy at room temperature, the number of components and their positions can be correlated although there might be some displacements due to temperature. The resolution is also different: 4 cm^{-1} in infrared and 5–8 cm^{-1} in resonance Raman. The mean positions of the components of the ketone carbonyl of spectra B–E (fig. 2 and table 1) are given in table 2. Except for the feature at 1713 cm^{-1} which is an ideal state component, the position and number of the other components are comparable to those observed by Lutz. In the monolayer situation that we have, there is no protein or lipid to bind the chlorophyll molecule, and the only other available molecule is water. In the associated ketone region, the different entities that we observe with the monolayer and that which Lutz has observed with the intact chloroplast could be the manifestation of different aggregation states of the chlorophyll molecules with water molecules. In the free ketone region, the different entities are assigned to free ketone vibrations in different dielectric milieux. So, in chloroplasts, we propose that some chlorophyll molecules are bonded to water molecules through hydrogen bonds with the ketone carbonyls and through coordinate bonds with the Mg atom in a fashion similar to that of the model presented in fig. 7.

3.1.4. Dry monolayer of Chl *a*

After heating the sample at 65°C under a vacuum of 0.1 Pa for 2 h, spectrum F (fig. 2) was taken, followed by spectra H–L, taken at time intervals. The position of the bands in the carbonyl region is given in table 1. In the hydroxyl region at approx. 3500 cm^{-1} (Fig. 2I) the small water band that was observed in spectrum E has almost disappeared; this is an indication that the more labile water molecule (of the third kind) has surely gone. The ester carbonyls which had formed one component at 1735 cm^{-1} before heat treatment (fig. 2E) have split into three components after heat treatment (fig. 2F). The maximum of the band is situated near 1737 cm^{-1} and is hardly perturbed in the subsequent spectra (G–L); the satellite bands situated near 1742 and near 1730 cm^{-1} show much greater variations, passing through some maxima, fading out and increasing in intensity. All these modifications are indicative that the dielectric milieu of the ester carbonyls is continually modified as a result of the mobility of the molecules.

In the carbonyl ketone region, the band with the maximum intensity is situated near 1697 cm^{-1} (fig. 2F) and this maximum is hardly modified with time after heat treatment. The situation is different for the satellite bands and the associated ketone carbonyl bands where considerable modifications are observed with time. The modifications that are observed in the free ketone region are assigned to dielectric perturbations that the free ketone experiences with time. The modifications in the associated ketone region, while less important than before the heat treatment, are still attributable to formation and rupture of hydrogen bonds between the ketone carbonyls and the hydroxyl groups.

In the skeletal region, the band is stabilized at 1620 cm^{-1} with small modifications with time. Below 1600 cm^{-1} (fig. 2I), small bands have replaced the absorption near 1550 cm^{-1} (fig. 2E). This activity arises from perturbation of the C=C, C=N skeletal bands which may result from interactions at the central Mg atom, the π-electron network or both. These modifications can be caused by the activity of the remaining water molecule (of the second kind) attached to the central atom. If this water molecule were not there, the monolayer would collapse into an inert structure.

3.1.5. Hydrated dry monolayer of Chl a

To determine the influence of water on a partially dehydrated monolayer, we have put the sample in a water atmosphere at 25°C for 10 h. After this period, the sample was put in the dry compartment of the spectrophotometer, and spectrum M (fig. 2) was taken, followed by spectra N–R. Spectrum Q taken 75 min after the water vapor bath shows, in the 3400 cm^{-1} region, a large band indicative of the presence of water molecules in the monolayer. The intensity of this band is greater than what has been observed previously; this is an indication that the nature of the interaction of the water with the chlorophyll is different from what has been obtained previously.

In the carbonyl region, the main ester carbonyl band at approx. 1737 cm^{-1} (fig. 2M–R) seems to be stabilized at this position with some slight fluctuation of the maximum from one spectrum to another. The satellite bands at 1742 and 1730 cm^{-1} of the preceding spectrum (fig. 2L) have gone and a new band has appeared at 1723 cm^{-1} (fig. 2M); 10 min later, this band is displaced to 1719 cm^{-1} (fig. 2N). In the following spectra (fig. 2O–Q) this band is not visible. On spectrum R, 145 min after the water vapor bath treatment, a satellite band has appeared at 1730 cm^{-1} displacing the maximum to 1740 cm^{-1}. These modifications are an indication of a varying dielectric milieu near the ester carbonyl groups as a result of the mobility of the chlorophyll molecules.

The ketone carbonyl region that had somewhat stabilized after the heat treatment becomes more active after the water vapor bath treatment. The maximum of the free ketone carbonyl band is still situated near 1697 cm^{-1} but the satellite bands near this maximum are increased, as is the activity of the associated ketone carbonyl bands as evidenced by the medium-intensity band at 1656 cm^{-1} in spectrum Q when previously it was less intense and afterward (Fig. 2R), it is also less intense. All this activity indicates that the water molecules try to penetrate the monolayer and form some bonds with whatever groups are available.

The skeletal band which was situated at 1622 cm^{-1} before the water vapor treatment (fig. 2L) is situated at 1605 cm^{-1} after (fig. 2M). This position is even lower than what was observed for a fresh multilayer (fig. 2B). After 10 min, the 1605 cm^{-1} band is modified into two components which are situated at 1621 and 1611 cm^{-1} (fig. 2N); 10 min later, they are situated at 1624 and 1609 cm^{-1} (fig. 2O), and a further 10 min later the two components fuse together to give one band at 1618 cm^{-1} (fig. 2P) which splits into three components 45 min later (fig. 2Q) which almost disappear in spectrum R. The skeletal C=O, C=N bands are directly affected by the incorporation and departure of water; these modifications arise, as before, from perturbation of the π-electron network and modifications at the central Mg atom.

3.1.6. Drying the hydrated dry monolayer of Chl a

Drying the hydrated monolayer of Chl *a* is done in two steps: the first step is carried out in a

Table 2

Positions (cm^{-1}) of the ketone carbonyl bands of Chl *a* in a fresh monolayer and in intact chloroplast

Relative intensity: m, medium; w, weak; v, very; e, extremely; sh, shoulder.

	Free keto C=O					Associated keto C=O			
Infrared spectroscopy of fresh monolayer of Chl *a* at 30°C [a]	1713ew sh	1701m	1697m	1689w sh	1679vw	1668vw	1664vw	1654vw	1638vw
Resonance Raman spectroscopy of *Botriopis alpina* at −248°C (35 K) [b]	-	1704sh	(1698)sh [c]	1691sh	1683m	1670sh	1660m	1651vw sh	1638ew

[a] Mean position of spectra B–E in fig. 2. Data taken from table 1.

[b] Resonance Raman data from Lutz[13]. *B. alpina* is a monocellular alga devoid of Chl *b*.

[c] This position is not mentioned in table 1 given in ref. 13 but on the spectrum we can see a shoulder of comparable intensity to that of the 1691 cm^{-1} component.

vacuum chamber (0.1 Pa) for 1.5 h at 25°C, and spectra S–V are taken at time intervals, followed by the second step where the drying is done at 65°C in the same vacuum chamber for 1.5 h, spectra W–ZZZ being taken at time intervals.

After the first step of drying, the hydroxyl band at approx. 3400 $\mathrm{cm^{-1}}$ has decreased in intensity (fig. 2V) but is still stronger than that in spectrum I. After the second step of drying (fig. 2ZZ), the hydroxyl band has increased in intensity. This indicates that water of the third kind has broken its coordinate bond with the Mg atom and has become available for hydrogen bonding with the carbonyls or the surface hydroxyls.

In the carbonyl region, after the first step of drying, the ester carbonyl band is broadened (fig. 2S), then a satellite band appears at 1731 $\mathrm{cm^{-1}}$ (fig. 2T), which is displaced to 1722 $\mathrm{cm^{-1}}$ (fig. 2U) and displaced further to 1727 $\mathrm{cm^{-1}}$ (fig. 2V). During the same time span, the maximum of the band which was situated at 1740 $\mathrm{cm^{-1}}$ before drying (fig. 2R) is situated at 1735 $\mathrm{cm^{-1}}$ after (fig. 2S), and was subsequently displaced to 1737 $\mathrm{cm^{-1}}$ (fig. 2T), then again at 1735 $\mathrm{cm^{-1}}$ (fig. 2U), and to 1740 $\mathrm{cm^{-1}}$ (fig. 2V). All these alterations are accompanied by some modifications in the broadness of the band. After the second step of drying, some sharp peaks situated at 1762, 1743 and 1727 $\mathrm{cm^{-1}}$ have appeared on spectrum W. These sharp peaks could be the manifestation of an abrupt phase change which recedes later on because the sharp peaks have broadened in the following spectrum (fig. 2X). At this stage, the whole ester carbonyl band is broadened; with time it sharpens and is accompanied by satellite features which come and go (spectra Y–ZZZ). All these modifications indicate that there is considerable activity in the vicinity of the ester carbonyls due to the interaction of water of the third kind that comes into the neighborhood of the ester groups.

In the ketone carbonyl region, the main band at 1697 $\mathrm{cm^{-1}}$ is not strongly perturbed after the first step of drying (fig. 2S) or subsequently (fig. 2T–V). In the associated ketone carbonyl region we observe a transient species at 1660 $\mathrm{cm^{-1}}$ in spectrum T which decreases in intensity in the following spectra. There are some other minor components appearing and disappearing that we can observe in these spectra. After the second step of drying, some sharp peaks have appeared in the spectrum taken immediately after drying (fig. 2W). These peaks are transformed into more intense and broader bands in spectrum X, disappear in spectrum Y, are reformed in spectrum Z accompanied by some displacement, and disappear again in spectra ZZ and ZZZ. The most intense ketone carbonyl band is displaced to 1700 $\mathrm{cm^{-1}}$ after heat treatment (fig. 2W), is split into two components in spectrum X and subsequently reunited into one component in the following spectra. In the vicinity of the free ketone carbonyl region, the modifications observed are assigned to perturbations of the dielectric environment caused by displacement of the molecules. The perturbations in the associated ketone region are due to the formation of hydrogen bonds of the ketone carbonyl with the water or hydroxyls of the surface.

In the skeletal region, the small bands in the 1620 $\mathrm{cm^{-1}}$ region are not greatly affected by the first step of drying (fig. 2S–V), but after the second step, the virtual nonexistence of the 1620 $\mathrm{cm^{-1}}$ components in spectrum V just before the second step gives rise to three medium-intensity components situated at 1630, 1616 and 1604 $\mathrm{cm^{-1}}$ (fig. 2W). These bands are modified subsequently as seen in spectra X–ZZZ.

The second step of drying removes water molecules of the third kind. This departure affects the central Mg atom, perturbing the C=C, C=N bands which will influence the π-electron network. Furthermore, the available water molecules will destabilize the monolayer arrangement, causing some shuffling of the molecules which will perturb the dielectric milieux of the functional groups. The available water molecules will form some hydrogen bonds with some carbonyls groups which will further modify the monolayer arrangement.

3.2. *Infrared spectra of Chl b*

To understand the influence of water on Chl *b* in monomolecular arrays, we have used the same procedure as for Chl *a*.

3.2.1. *Multilayer of Chl b*

The spectrum of a freshly prepared multilayer of Chl *b*, taken 20 min after the end of deposition, is presented in fig. 8B. After 120, 140 and 160 min spectra C, D and E were taken. After heating the sample at 75°C for 2.5 h under vacuum (0.1 Pa), spectrum F was taken. The sample was then exposed to water vapor at 24°C for 9 h and spectrum G was taken followed by spectra H, I and J taken 45, 75 and 105 min after. The sample was then put in a vacuum (0.1 Pa) for 15 h at 24°C and spectrum K taken.

The spectrum of the dry multilayer of Chl *b* (fig. 8F) is essentially the same as that given in part II [1] before the dioxane vapor treatment, and the assignments given in table 3 follow which were given previously. The component at 1736 cm^{-1} is the ester carbonyl band, that at 1699 cm^{-1} the free ketone carbonyl band, that at 1664 cm^{-1} a mixture of the coordinated ketone carbonyl band with the free aldehyde carbonyl band, that at 1606 cm^{-1} a mixture of the coordinated aldehyde carbonyl band with the skeletal C=C, C=N band, and those at 1545 and 1519 cm^{-1} the skeletal C=C, C=N bands. The position of the bands of the other spectra in fig. 8 is placed in table 3 according to this division.

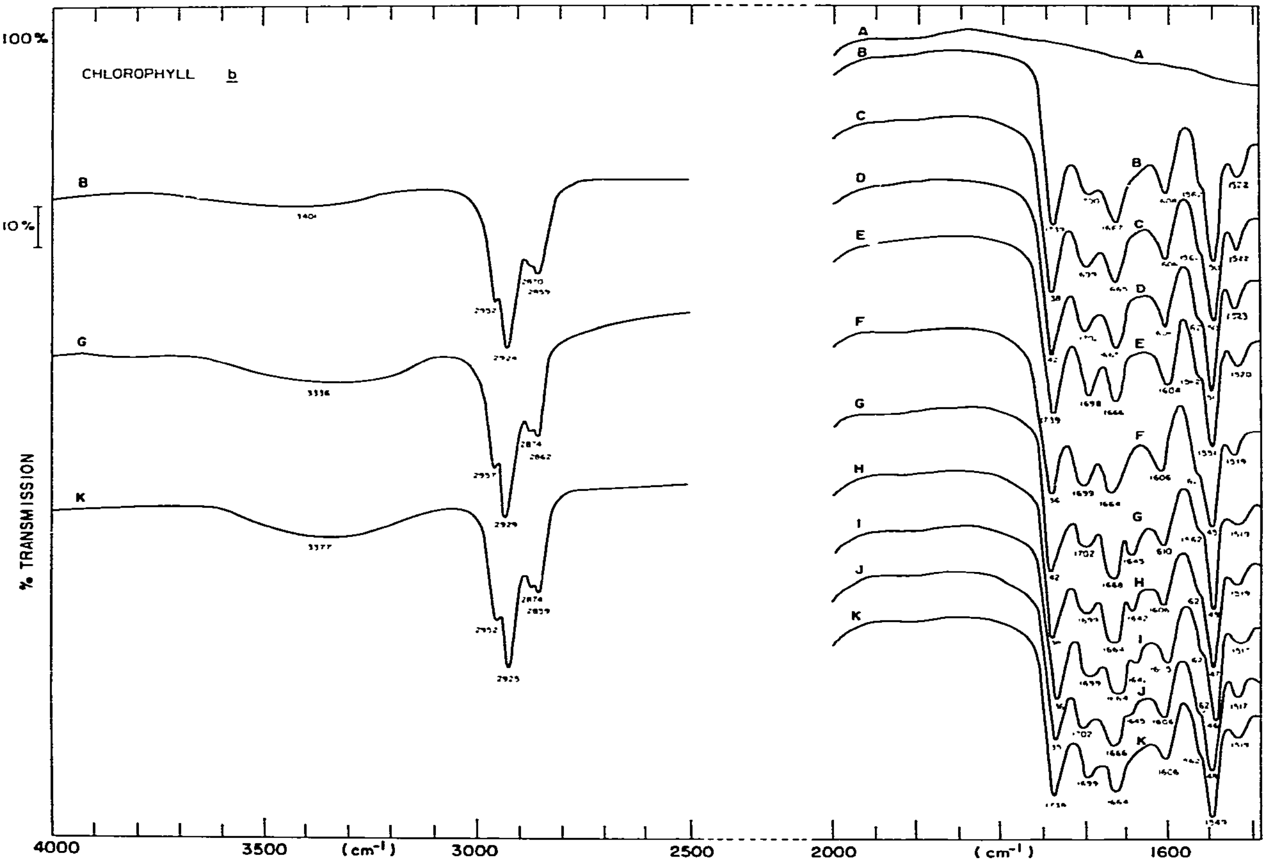


Fig. 8. Infrared spectra of the effect of water vapor on a Chl *b* multilayer (2×41 layers on Ge). (A) Background; (B) sample, 20 min after deposition; (C) 120 min after; (D) 140 min after; (E) 160 min after; (F) after heating at 75°C under vacuum (0.1 Pa) for 2.5 h and after cooling down; (G) after 9 h in water vapor at 24°C (≈ 30 kPa); (H) 45 min after; (I) 75 min after; (J) 105 min after; (K) after sample was under vacuum (0.1 Pa) for 15 h at 24°C.

If we look first at the spectrum of the multilayer after the water vapor treatment (fig. 8G), we notice that the free ketone carbonyl band is decreased in intensity and slightly displaced to 1702 cm^{-1}, and that a new band has appeared at 1645 cm^{-1}. The other bands are not greatly affected by the water vapor treatment except for some small displacements of their maxima. With time, the intensity of the 1645 cm^{-1} component decreases while that of the 1702 cm^{-1} component increases (fig. 8H–K). The 1645 cm^{-1} component is assigned to the ketone carbonyl, associated with water. As the water departs in the dry compartment of the spectrometer, the free ketone carbonyl band is restored. With the water vapor treatment, the O-H stretch band has increased in intensity, giving a structureless band situated at approx. 3336 cm^{-1} (fig. 8G) which decreases slightly with time. This band is clearly associated with water in the multilayer.

When the spectrum of the multilayer, after the water vapor treatment, (fig. 8G) is compared with the 'instable' Chl *b* hydrate of Ballschmitter and Katz [8], we notice that the position of the bands is almost coincident, especially the ketone carbonyl bands where the authors have observed a shoulder at approx. 1700 cm^{-1}, a band at 1670 cm^{-1}, and another at 1645 cm^{-1}. On standing in the infrared spectrophotometer, the hydrated film of Chl *b* loses the 1645 cm^{-1} absorption and the approx. 1700 cm^{-1} absorption is increased. This situation is similar to what we have observed (fig. 8G–K), therefore the aggregation state of both systems must be very similar.

For the spectrum of the freshly prepared multilayer of Chl *b* taken 20 min after deposition (fig. 8B) a small free ketone carbonyl band is observed at approx. 1700 cm^{-1}. With time, this band increases in intensity (fig. 8C–E). The bands at approx. 1667 and approx. 1606 cm^{-1} are slightly modified with time: they become sharper with a slight intensity decrease and the trough in between these two bands is also modified. After heating under vacuum (fig. 8E), the intensity of the free ketone carbonyl is maximal; the band now situated at 1664 cm^{-1} has broadened a little while the 1606 cm^{-1} component has sharpened a little. The intensity of the ester carbonyl band at approx. 1740 cm^{-1}, and those for the skeletal vibrations at approx. 1550 and approx. 1520 cm^{-1} are not greatly affected with time; only their positions are slightly modified. These displacements can be attributed to the varying dielectric milieux surrounding these groups. In the O-H stretch region, there is a wide, low-intensity, and structureless band situated at approx. 3400 cm^{-1} (fig. 8B). If we were to extrapolate our data to the period immediately after deposition (time zero), the free ketone carbonyl band at approx. 1700 cm^{-1} would be absent, the 1667 and 1608 cm^{-1} bands slightly more intense, and the trough in between these two bands would be less pronounced but it is doubtful whether a distinctive feature at approx. 1645 cm^{-1} would be present, such as that appearing on spectrum G.

If we compare the spectrum of a freshly prepared multilayer of Chl *b*, and the subsequent spectra (fig. 8B–E) with those obtained after the effect of water vapor on the dried multilayer (fig. 8G–K), we notice that the intensity of the O-H stretch band at approx. 3400 cm^{-1} is less pronounced in the former than in the latter. The free ketone carbonyl at approx. 1700 cm^{-1} behaves approximately the same way in both cases; the band at approx. 1665 cm^{-1} behaves a little differently; the band at 1645 cm^{-1} is quite readily visible (fig. 8G–J) in the second case and decreases steadily with time; in the first case (fig. 8B–E) there is a broad shoulder at approx. 1645 cm^{-1} that decreases with time; the band at 1606 cm^{-1} is a little different in both cases; and the other bands at approx. 1550 and approx. 1520 cm^{-1} are not greatly affected throughout, except for some small displacements of the bands maxima. Although there are some similarities between these two cases, there are some annoying differences, notably the O-H stretch band and the 1645 cm^{-1} band. The aggregation structure must therefore be slightly different in both cases, and since the 1645 cm^{-1} component has been assigned to the ketone carbonyl associated with water and the approx. 3400 cm^{-1} band is in the region of the O-H stretch, the differences between the two species must be due to the organization of water molecules in the multilayer.

If we compare the spectrum of the freshly pre-

Table 3

Wave number positions (cm^{-1}) of Chl *b* in the carbonyl region

Spectrum and description		Ester C=O			
	1820–1760	1760–1745	1745–1737	1736–1728	1727–1716
1. Multilayer					
(B) 25 min after deposition			1739		
(C) 120 min after			1738		
(D) 140 min after			1742		
(E) 160 min after			1739		
(F) Vacuum, 75°C, 2.5 h				1736	
(G) Water vapor, 24°C, 9 h			1742		
(H) 45 min after			1738		
(I) 75 min after				1736	
(J) 105 min after				1735	
(K) Vacuum, 25°C, 15 h			1738		
2. Monolayer					
(B) 15 min after deposition				1733	1717
(C) 25 min after		1756 1747	≈1740	1735	
(D) 35 min after				1734	1724
(E) 70 min after				1735	
(F) Vacuum, 65°C, 1.5 h				1736	≈1721sh
(G) 10 min after		1755	≈1740sh	1732	
(H) 20 min after			1738	1730	
(I) 40 min after			1737	≈1734	≈1716sh
(J) Water vapor, 25°C, 25 h			1734		1721
(K) 10 min after			1737	1728	1719
(L) 20 min after		1751	1744	1730	
(M) 50 min after	1796	1758		1734	
(N) Vacuum, 65°C, 1.5 h		1757		1736	
(O) 10 min after			1738	1729	
(P) 20 min after			1736	1731	
(Q) 55 min after	1791 1771			1734	
Mean ± S.D.		1754 ± 4	1739 ± 3	1733 ± 3	1720 ± 3

Table 3 (continued)

Spectrum and description	Free keto C=O					Associated keto C=O, Free aldehyde C=O			
	1715–1705	1704–1700	1699–1695	1694–1686	1685–1677	1676–1668	1667–1658	1657–1650	1649–1637
1. Multilayer									
(B) 25 min after deposition		1700					1667		
(C) 120 min after			1699				1665		
(D) 140 min after		1702					1665		
(E) 160 min after			1698				1666		
(F) Vacuum, 75°C, 2.5 h			1699				1664		
(G) Water vapor, 24°C, 9 h		1702				1668			1645
(H) 45 min after			1699				1664		1642
(I) 75 min after			1699				1664		1642
(J) 105 min after		1702					1666		1645
(K) Vacuum, 25°C, 15 h			1699				1664		
2. Monolayer									
(B) 15 min after deposition	≈ 1710	1700		1686		≈ 1676	1663	1654	
(C) 25 min after	1706sh	1700		1688	1682	1669	≈ 1664sh	1651	1647
(D) 35 min after		1703			1685		1660		1648
(E) 70 min after		1703		1692			1659		
(F) Vacuum, 65°C, 1.5 h		1701	1697				1662	1653	≈ 1641sh
(G) 10 min after		1704		1688	1677		1658		1645
(H) 20 min after		1702	1695		1680		1662	1655	1641
(I) 40 min after	≈ 1707sh		≈ 1698sh	1692	≈ 1686	1670	1661		1643
(J) Water vapor, 25°C, 25 h		1701	1698		1684		1664	1659	
(K) 10 min after	1706			1691	1685	1671	1659		
(L) 20 min after		1700		1691		1672	1663	1655	1644
(M) 50 min after			1699				1662		
(N) Vacuum, 65°C, 1.5 h	1708			1693			1658		1641
(O) 10 min after	1708	1702		1694		1671	1659		1642
(P) 20 min after		1701		1694		1668	1663	1651	
(Q) 55 min after		1702		1692	1684	1676	1662	1650	
Mean ± S.D.	1707 ± 2	1702 ± 1	1698 ± 1	1691 ± 3	1683 ± 3	1671 ± 3	1663 ± 3	1654 ± 3	1644 ± 3

Table 3 (continued)

Spectrum and description	Skeletal C=C, C=N (Associated aldehyde, C=O...)							
	1636–1628	1627–1616	1615–1604	1603–1581	1580–1561	1560–1541	1540–1520	1520–1510
1. Multilayer								
(B) 25 min after deposition			1608		1562sh	1550	1522	
(C) 120 min after			1606		1562sh	1550	1522	
(D) 140 min after			1606		1562sh	1551	1523	
(E) 160 min after			1604		1562sh	1551	1520	
(F) Vacuum, 75°C 2.5 h			1606		1562sh	1545		1519
(G) Water vapor, 24°C, 9 h			1610		1562sh	1549		1519
(H) 45 min after			1606		1562sh	1547		1519
(I) 75 min after			1605		1562sh	1546		1517
(J) 105 min after			1606		1562sh	1548		1517
(K) Vacuum, 25°C, 15 h			1606		1562sh	1549		1519
2. Monolayer								
(B) 15 min after deposition	1636 1628		1610					
(C) 25 min after	1636	1618	1608					
(D) 35 min after			≈1605					
(E) 70 min after	1628		1606		≈1565sh	1547	1521	1511
(F) Vacuum, 65°C, 1.5 h			1606					
(G) 10 min after	1630		1613sh, 1606					
(H) 20 min after		1621	1605					
(I) 40 min after		1621	1606	1584	≈1564sh	1547	1524	1516
(J) Water vapor, 25°C, 25 h	1633		1610					
(K) 10 min after		1619	1611					
(L) 20 min after	1629		1613					
(M) 50 min after	1637	1622	1606	1585	≈1563sh	1547		1514
(N) Vacuum, 65°C, 1.5 h	1628		1605					
(O) 10 min after	1630	1617						
(P) 20 min after	1638	1622	1605					
(Q) 55 min after		1622	≈1604		1566	1549	1528	≈1518sh 1508
Mean ± S.D.	1632±4	1620±2	1607±3	1585±1	1563±1	1548±2	1523±3	1516±4

pared multilayer of Chl *b* and those obtained after time intervals (fig. 8B–E) with the spectrum of a freshly prepared multilayer of Chl *a* and those obtained after time intervals (fig. 3B–E in ref. 3), we notice that the intensity of the free ketone carbonyl band, approx. 1700 cm^{-1} in the former and approx. 1695 cm^{-1} in the latter, increases in the same fashion. The difference in the position of the bands is due to a different dielectric milieu. The Mg atom-coordinated keto carbonyl band is situated at approx. 1656 cm^{-1} in the multilayer of Chl *a*; this band is maximal at time zero after the preparation, and the free keto carbonyl band at approx. 1700 cm^{-1} is minimal. With time, the coordinated keto carbonyl band is decreased while the free keto carbonyl band is increased. For the multilayer of Chl *b* (fig. 8B–D), the band situated at approx. 1667 cm^{-1} shows some decrease in intensity but not as pronounced as that for Chl *a*. The band situated at 1608 cm^{-1} shows also some slight decrease in intensity in Chl *b* while there is little modification in the Chl *a* multilayer. The trough in between the approx. 1667 and approx. 1608 cm^{-1} bands shows some modifications in the Chl *b* multilayer; there is no such modification in the Chl *a* multilayer. In the Chl *a* multilayer, we have postulated that there are two water molecules associated with each Chl molecule, and that the departure of one water molecule will make the multilayer buildup collapse, breaking many keto CO---Mg polymeric links. For the multilayer of Chl *b*, we postulate also the presence of water molecules in the multilayer and that the departure of some water molecules will make the multilayer buildup collapse, freeing some keto carbonyls and some aldehyde carbonyls that were associated with some other molecules, either of water or of some other Chl *b*. The association of the water molecules with Chl *b* molecules will be different from what has been postulated for Chl *a*, and must be due to the presence of the aldehyde group.

If we look at the buildup of a multilayer of Chl *b*, the ketone carbonyl and aldehyde carbonyl groups will alternately be in contact with the Mg atom of the adjacent Chl *b* molecule, and coordination will take place alternately with both groups. This liberates either a ketone carbonyl or an aldehyde carbonyl which can be associated with water molecules. These water molecules will therefore be associated on one side with the carbonyl and on the other side with the Mg atom of adjacent Chl *b* molecules. The proposed organization is presented in fig. 9. For a freshly prepared multilayer of Chl *b*, the ketone carbonyl and aldehyde carbonyl are alternately attached to the Mg atom of the Chl *b* molecules. The other carbonyl on each Chl *b* molecule is hydrogen bonded to water molecules which are coordinate bonded to the Mg atom of neighboring Chl *b* molecules. From section 3.2.3,

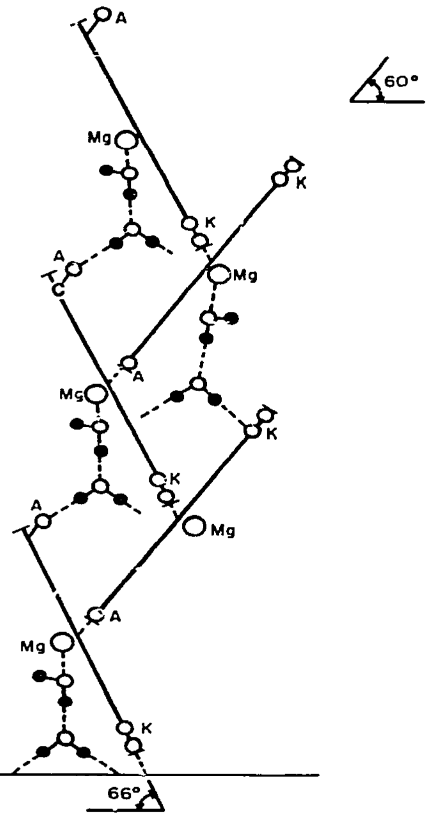


Fig. 9. Schematic representation of the Chl *b* multilayer buildup. The angle of tilt of the first layer is approx. 66°, the others are approx. 60°. A, aldehyde carbonyl; K, ketone carbonyl.

we find the first layer to be tilted at an angle of 66°; the next layers are at an angle of 60° to ensure that the buildup is made. As the more labile water molecules depart the buildup collapses, breaking many polymeric links. 20 min after the end of deposition, some water molecules would have departed from the multilayer and the situation would be as follows: the liberated ketone carbonyls would give rise to the 1700 cm^{-1} component which increases in intensity with time, indicating that more ketone carbonyls are being liberated. This band soon reaches a maximum (fig. 8E and F) which is, however, not intense enough to account for all the ketone carbonyls in the multilayer. Therefore, there are some coordinated ketone carbonyls in the 1664 cm^{-1} component which also contains the free aldehyde carbonyls. This band and the shoulder at lower wave number can also contain the ketone carbonyl associated with water. This association, as we have seen for the monolayer of Chl *a*, need not give rise to a single value at approx. 1645 cm^{-1} but can result in many components absorbing in the 1675–1628 cm^{-1} range. The aldehyde group associated with either the Mg atom of neighboring molecules or with water molecules can absorb broadly in the range from approx. 1640 to 1581 cm^{-1}. With time, many associated carbonyl aldehyde bonds will break, giving rise to free aldehyde carbonyl absorption at approx. 1665 cm^{-1}, and since some coordinate ketone bonds are broken, the intensity of the approx. 1665 cm^{-1} remains practically constant.

3.2.2. Monolayer of Chl b

The spectrum of a freshly prepared monolayer of Chl *b*, taken 15 min after the end of deposition, is shown in fig. 10B. Leaving the sample in the dry compartment of the spectrometer, spectra C, D and E were taken 25, 35 and 70 min after the end of deposition. After heating the sample under vacuum (0.1 Pa) at 65°C for 1.5 h, spectrum F was taken. The subsequent spectra G to I were taken 10, 20 and 40 min after. The sample was then put in a water vapor bath at 25°C for 25 h and then spectrum J was taken followed by spectra K, L and M taken 10, 20 and 50 min after. The sample was then heated at 65°C under vacuum (0.1 Pa) for 1.5 h, and spectrum N taken, followed by spectra O, P, Q and R taken 10, 20, 55 and 102 min after. The position of the bands is given in table 3. The major divisions are those used for the multilayer, and subdivisions list the bands whose positions reflect the vibration of a functional group in the same dielectric environment.

The divisions given in table 3 for Chl *b* follow those given in table 1 for Chl *a* with minor modifications to take into account the additional aldehyde carbonyl group. The region of the ketone carbonyl and aldehyde carbonyl extends from 1715 to 1580 cm^{-1}. In this region, there are the free and associated aldehyde carbonyls, and from 1627 to 1580 cm^{-1} one of the skeletal C=C, C=N vibrational bands. Due to the varying dielectric milieux, each group can manifest itself by many components that can overlap with each other, giving rise to accidental degeneracy. Because of these phenomena, the 1715–1580 cm^{-1} region is much more complex than that observed for Chl *a*. Nevertheless, if we accept that the intensity pattern should follow approximately the intensity pattern of Chl *a*, one would obtain the following divisions: the free ketone carbonyl bands are situated in the region 1715–1676 cm^{-1}, the most intense component of this group being situated around 1700 cm^{-1}; the associated ketone carbonyl gives rise to bands whose intensities are much weaker than that of the free ketone carbonyl and are situated in the region 1675–1637 cm^{-1}; the free aldehyde carbonyl bands are situated in the same region but their intensity should be greater than that of the associated carbonyl bands so that the most intense bands in this region should be assigned to the free aldehyde carbonyl, the principal feature of this group being situated at approx. 1661 cm^{-1}; the associated aldehyde carbonyl bands are situated in the region 1637–1581 cm^{-1}, the aldehyde carbonyl being associated with water by weak hydrogen bonds and when the water departs these bonds are broken, modifying the bands in this region and those in other regions.

To illustrate the greater perturbations that the Chl *b* monolayer undergoes with modification in the aggregation state, we look at the free ketone carbonyl band. In Chl *a*, the main feature of this band is approximately stable near 1697 cm^{-1} with low-intensity varying satellite bands situated on each side of the main feature. In Chl *b*, the intensity of the main feature of the free ketone carbonyl oscillates between three positions: 1702, 1698 and 1691 cm^{-1}. This is an indication that the environment of this functional group is much more perturbed than in the case of Chl *a*. The extent of these modifications can be explained by the rupture of the associated aldehyde carbonyl bond that causes a shuffling of the molecules placing the carbonyl groups in different environments.

The skeletal C=C, C=N bands are situated in the 1636–1500 cm^{-1} region. In this region, the approx. 1548 cm^{-1} component is fairly stable and to a lesser degree so is the approx. 1607 cm^{-1} component. The portion of the latter that is assigned to skeletal vibrations should behave approximately in the same manner as the approx. 1548 cm^{-1} component. The major vibration of this band is due to the associated aldehyde carbonyl that has some varying components in this spectral range. The fact that the skeletal bands are hardly affected by modifications in the aggregation state of the monolayer is an indication that the π-electron network which would affect the C=C, C=N vibrations is not significantly influenced by the monolayer organization and their subsequent modifications with changes in the aggregation state. This situation is different from what we have seen for Chl *a*.

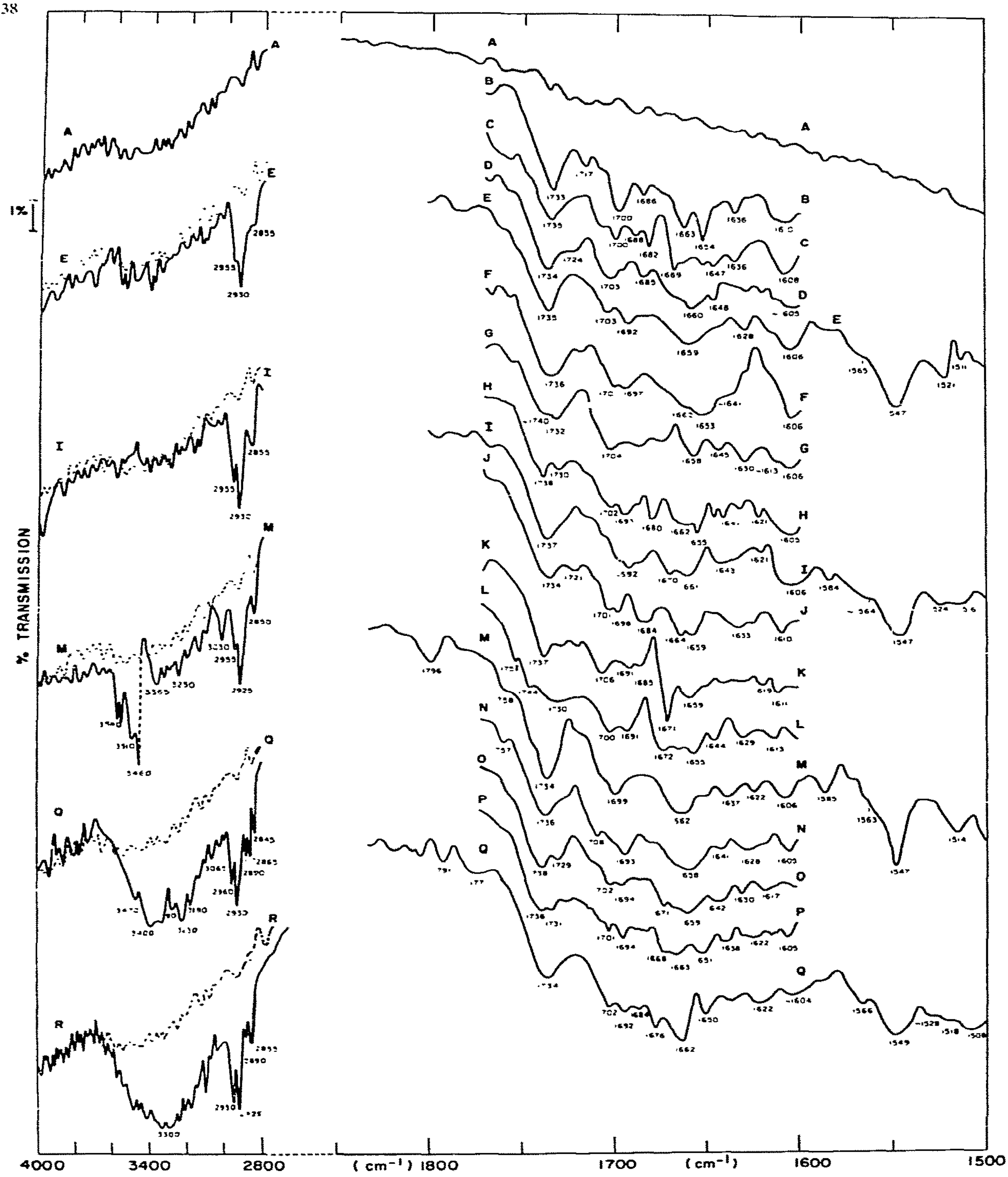
The sample was deposited onto a Ge MIR plate whose background spectrum is given in fig. 10A. Except for a broad structureless band situated at approx. 3400 cm^{-1}, the spectrum is free of absorption bands. The band at approx. 3400 cm^{-1} is assigned, as for the Chl *a* monolayer, to hydroxyl groups on the surface of the MIR plate. Therefore, the surface of the Ge MIR plate before deposition is the same as for Chl *a* and is represented schematically in fig. 3A.

From the observations we have made on Chl *a*, the organization of the monolayer at the air/water interface is the same as that of a freshly prepared monolayer on a hydrophilic plate. The same situation will also prevail for Chl *b*. We will now look at the pressure-area isotherm of Chl *b* to determine the distance between the Chl *b* molecules, the angle of tilt of the porphyrin planes, and the position of the water molecules in between. This information will give us a model for the aggregation of Chl *b* at the air/water and air/solid interfaces.

The surface pressure-area isotherm of pure Chl *b* at the nitrogen/water interface taken at 20.0°C is shown in fig. 5. The deposition is done at a pressure, π, of 20 mN m^{-1}. At this pressure, the area covered by one molecule of Chl *b* is 81.1 Å^2 ($A_{\pi=20} = 81.1$ Å^2 molecule^{-1}). The surface of the porphyrin plane above the surface of water is the same as that for Chl *a*, 198.7 Å^2. At $\pi = 20$ mN m^{-1}, the angle of tilt of the porphyrin plane is 66° and the distance between the planes 6.2 Å. This distance is shorter than that for Chl *a*, and this situation is attributed to the presence of the aldehyde group where the oxygen can make a hydrogen bond with one hydrogen of the water dimer. The schematic organization of the water in the monolayer arrays is given in fig. 11.

With a distance of 6.2 Å between the porphyrin planes, the distance between Mg and O_6 atoms is calculated to be 6.5 Å. Projected on the *XY* plane this distance is 6.5 cos 27.3° = 5.8 Å. As for Chl *a*, the water that can go in between the porphyrin planes is water of the first kind, that is to say the bonding between the water molecules and that between the water and Chl *b* molecules are of the gas-phase type. Because the distance of 6.5 Å between the Mg and O_6 atoms is shorter than the analogous distance in Chl *a*, it is necessary to tilt the dihedral angle, θ_A, to a value of approx. 58° in order to accommodate the water dimer in this distance. The structure of the water dimer is the same as that given in fig. 4A, and the position of the water dimer in the monolayer of Chl *b* would

Fig. 10. Infrared spectra of the effect of water vapor on a Chl *b* monolayer (2×1 layer). (A) Background; (B) sample, 15 min after deposition; (C) 25 min after; (D) 35 min after; (E) 70 min after; (F) after heating at 65°C under vacuum (0.1 Pa) for 1.5 h; (G) 10 min after; (H) 20 min after; (I) 40 min after; (J) after 25 h in the water vapor bath at 25°C (3.2 kPa); (K) 10 min after; (L) 20 min after; (M) 50 min after; (N) after heating at 65°C under vacuum (0.1 Pa) for 1.5 h; (O) 10 min after; (P) 20 min after; (Q) 55 min after; (R) 102 min after. The dashed lines are spectrum A reproduced.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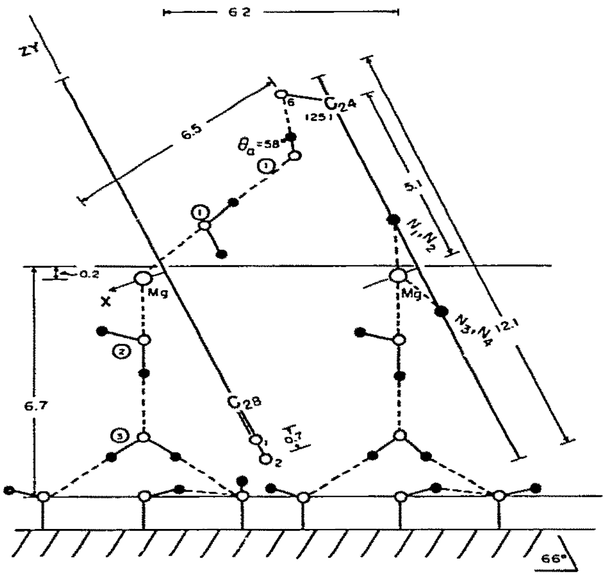

Fig. 11. Schematic representation of the Chl *b* molecules at the air/water interface incorporating the water molecules. The labels 1, 2 and 3 indicate water of the first, second and third kind (O_1 and O_2 are hydrogen bonded to some hydrogen atoms that are not shown). Distances are in Å; angle of tilt is 66° at $\pi = 20$ mN m^{-1}. The Mg---O_6 distance projected on the *xy* plane is 6.5 cos 27.3° = 5.8 Å.

be that given in fig. 11. With a dihedral angle of approx. 58° and a hydrogen bond between one hydrogen of one water molecule with O_6 of the Chl *b* molecule, the hydrogen bond between these two molecules would be a little stronger than in Chl *a*. This extra strength would be distributed along the chain from the Mg to O_6 because of the cooperative effort in a chain-like situation such as this.

Under a pressure of 20 mN m^{-1} at the air/water interface, the porphyrin planes of Chl *b* are tilted at an angle of 66°. The Mg atom, situated 0.43 Å out of the plane, points toward the water surface, and the perpendicular distance between the Mg atom and the water surface is 6.5 Å, a distance sufficient to place two water molecules in a fashion similar to that of Chl *a*, except that the distance is a little longer, making the hydrogen bonds a little weaker.

A schematic representation of the aggregation state of Chl *b* at the air/water interface is given in fig. 11. In this representation, the water dimer situated between the Mg atom of one Chl *b* molecule and the O_6 atom of the other constitutes water of the first kind. This kind of water will depart easily in a competing situation. The other two water molecules, on the other side of the porphyrin plane and situated between the Mg atom and the water of the subphase, constitute the water of the second and third kinds linked together by a hydrogen bond. The oxygen of water of the second kind makes a strong coordinate bond with the Mg atom. This kind of water will be the one most difficult to remove. The hydrogens of water of the third kind are linked to the oxygen of the water surface by weak hydrogen bonds.

Since the surface of a hydrophilic plate is similar to that of the water trough, the organization of the Chl *b* molecules on such a plate will be similar.

3.2.3. Fresh monolayer of Chl *b*

In fig. 10B, the spectrum of a freshly prepared monolayer of Chl *b* taken 15 min after the end of deposition is presented. This spectrum is followed by spectra C–E taken at time intervals after spectrum B. The Ge plate with one layer of Chl *b* on both sides of the plate is maintained in the dry compartment of the spectrometer.

In the ester carbonyl region in spectrum B (fig. 10) there is one main band situated at 1733 cm^{-1} and a weaker band situated at 1717 cm^{-1}. The main band is fairly constant from spectrum B to E. The satellite band disappears in spectrum C but weaker bands appear at approx. 1747 and approx. 1740 cm^{-1}. These bands are decreased in intensity in spectrum D and a shoulder appears at approx. 1724 cm^{-1} which disappears in spectrum E. These modifications are an indication that, although most of the ester groups are not affected during these spectra, some ester groups are affected and this must occur through interactions with the surface hydroxyls, since they are close to them. These interactions are weak, since these satellite bands are continually modified.

In the free ketone carbonyl region (1715–1676 cm^{-1}) of the spectrum of a freshly prepared monolayer of Chl *b* taken 15 min after deposition (fig. 10B), there is one main feature situated at

1700 cm^{-1}. The satellite components at approx. 1710, 1686 and approx. 1676 cm^{-1} are a manifestation that some ketone carbonyls are in different dielectric milieux. The relative intensities and pattern of intensities of these bands are almost the same as those of the comparable bands of Chl *a* (fig. 2B). In subsequent spectra (fig. 10C–E), these bands are modified: in spectrum C, the ketone carbonyls form four components of almost equal intensities; in spectrum D, they form one main band at 1703 cm^{-1} and a weaker one at 1685 cm^{-1}; in spectrum E, they form two bands of almost equal intensities. In Chl *a*, there are as many components as in Chl *b* but the intensity pattern of the spectra after spectrum B is different. An explanation of these spectra is that from time zero up to the time of spectrum B, the situation of the ketone carbonyls of Chl *b* is similar to that with Chl *a* but thereafter different. This is due to the rupture of the associated aldehyde carbonyl bond which makes a stronger bond than the π-hydrogen bond of Chl *a*, so that rupture of the stronger bond causes more perturbations than that of a weaker bond such that the ketone carbonyls of Chl *b* will be placed in a wider variety of situations than those of Chl *a*.

In the region 1675–1637 cm^{-1} of the spectrum of a freshly prepared monolayer of Chl *b* taken 15 min after deposition (fig. 10B), the band at 1663 cm^{-1} has been assigned to the principal component of the free aldehyde carbonyl. This position is the same as that of Chl *b* in a tetrahydrofuran solution [12] where all chlorophyll-chlorophyll interactions have been eliminated. The relative intensity of the band is also comparable to that in a polar solution. The band at 1654 cm^{-1}, which is equal in intensity to that at 1663 cm^{-1}, is assigned to a composite of the associated ketone carbonyls and to the remaining free aldehyde carbonyls which are in a different dielectric milieu than the 1663 cm^{-1} band.

The band at 1636 cm^{-1}, the shoulder at approx. 1628 cm^{-1}, and part of the band at 1610 cm^{-1} (fig. 10B) have been assigned to the associated aldehyde carbonyl because their positions relative to the free aldehyde carbonyl at 1663 cm^{-1} are approximately the same as those of the associated ketone carbonyl bands of Chl *a* relative to the free ketone carbonyl one. The relative intensities are also comparable. With time, there are some modifications of these bands (fig. 10C–E); the overall intensity is decreased with modifications of the intensity pattern from one spectrum to the other. At time zero, when the association of the water molecules to the aldehyde carbonyl is maximal, the organization of the molecules is that given in fig. 11. After some time in the dry compartment of the spectrometer, the most easily removed water, that of the first kind, will depart breaking the hydrogen bond between the water molecule and the oxygen of the aldehyde. This perturbation will increase the intensity of the bands of the free aldehyde carbonyl in the 1675–1637 cm^{-1} region, and will also destabilize the chlorophyll molecules, placing the ketone and aldehyde carbonyls in a wide variety of situations. The manifestation of this phenomenon will be the formation of many components of varying positions and intensities as the bonds are broken and reformed depending on the steric conditions of the molecules.

3.2.4. Dry monolayer of Chl b

After heating the sample at 65°C under a vacuum of 0.1 Pa for 25 h, spectrum F (fig. 10) was taken followed by spectra G–I taken at time intervals. The position of the bands in the carbonyl region is given in table 3. In the hydroxyl region, the absorption that was almost coincident with the background before heat treatment (fig. 10E) has decreased slightly after (approx. 3500 cm^{-1} in fig. 10I). This is interpreted as the departure of some water molecules of the third kind from their original position (see fig. 11).

The ester carbonyls which had formed one component at 1735 cm^{-1} with small shoulders on the high-frequency side (fig. 10E) have broadened after heat treatment and a shoulder has appeared at approx. 1721 cm^{-1} (fig. 10F). In subsequent spectra, the stronger component is split into two (fig. 10G and H), and fused into one (fig. 10I); there are also some weaker components appearing and disappearing. This situation is similar to that for Chl *a* and is interpreted as being a result of the mobility of the molecules which modifies continually the environment of the ester carbonyls.

In the ketone carbonyl and aldehyde carbonyl region, there is a big decrease in the intensity of the absorption in the associated aldehyde carbonyl region at approx. 1625 cm^{-1} (fig. 10F) and an increase in the free aldehyde carbonyl region at approx. 1653 cm^{-1}. This is a consequence of the departure of the remaining water of the first kind (see fig. 11) which frees the aldehyde carbonyl. The free ketone carbonyl components are also modified: there is a slight increase in intensity of the bands at 1701 and 1697 cm^{-1}. This is interpreted as the consequence of liberation of some associated ketone carbonyls. With time, there are considerable modifications of the intensities of many components in this region (fig. 10G–I). These modifications are the result of the shuffling of the molecules and also of the reformation of the associated aldehyde carbonyls as we can see an increase in the absorption in the approx. 1620 cm^{-1} region. This increase could be due to the formation of a coordinate bond between the aldehyde of one chlorophyll molecule with the Mg atom of another chlorophyll molecule as one molecule is tilted toward the other. This possibility is not considered to be the case here because of steric difficulties and because if this band were formed it would give rise to a stable bond which would give a stable band in the vicinity of 1620 cm^{-1}, this is not the case as we can see in the subsequent spectra. The other possibility is the formation of hydrogen bonds with the liberated water of the third kind; this association would lead to transient species giving rise to a changing intensity pattern, and this is what we observe in spectra G–I.

3.2.5. Hydrated dry monolayer of Chl b

The influence of water on the partially dried monolayer of Chl *b* was studied by placing the sample in a water atmosphere at 25°C for 24 h. After this period, the sample was put in the dry compartment of the spectrophotometer and spectrum J (fig. 10) was immediately taken followed by spectra K–M. Spectrum M taken 50 min after the water vapor bath shows, in the hydroxyl region, an absorption with many components in the 3600–3200 cm^{-1} region. This absorption is attributed to the presence of water of many transient organizations. The dashed line at 3460 cm^{-1} indicates the position where the spectrophotometer was stopped because there were too many fluctuations on the pen. The fact that the absorption is very different on each side of this dashed line is the consequence of a phase transition taking place at that moment. In spectrum M, the CH bands in the 3100–2800 cm^{-1} range show some modifications compared to spectrum I. This is attributed to displacements of the phytyl chain which would place some C-H groups in different dielectric milieux.

After the water vapor treatment the ester carbonyls show a small band at 1721 cm^{-1} beside the main absorption at 1734 cm^{-1} (fig. 10J). In the following spectrum (fig. 10K), the main absorption is shifted to 1737 cm^{-1} and the small component has given rise to two components at 1728 and 1710 cm^{-1}, and then on the next spectrum (fig. 10L), all these components are fused together to give a large absorption with its maximum at approx. 1730 cm^{-1} and a few satellite bands on the high-frequency side. In the next spectrum (fig. 10M), this absorption becomes sharper with its maximum at approx. 1734 cm^{-1} and a few faint satellite bands on each side. Because of the mobility of the molecules, the carbonyls in different dielectric milieux will give rise to many components. There is also the possibility of the formation and deformation of an association of the ester carbonyls with the water molecules, and the large component at approx. 1730 cm^{-1} would be the manifestation of such an entity.

In the ketone carbonyl and aldehyde carbonyl region, there is an increase in intensity of the absorption at approx. 1633 cm^{-1} (fig. 10J) due to the formation of an association of the aldehyde with water. The absorption in the associate aldehyde carbonyl region is not drastically modified in subsequent spectra (fig. 10K–M); this is an indication that the associated aldehyde carbonyl is fairly stable. The situation is different in the ketone carbonyl region. Before the water vapor treatment there is one band situated at 1692 cm^{-1}, and after the treatment, the band is split into two components situated at approx. 1700 and 1684 cm^{-1} (fig. 10J). These components are further modified in the following spectrum (fig. 10K) where a sharp component has appeared at 1671 cm^{-1}. This component broadens in the following spectrum (fig. 10L) with modifications in the approx. 1700 cm^{-1} region. In the next spectrum (fig. 10M), the sharp component has almost disappeared and the components in the approx. 1700 cm^{-1} region have fused together to give one band at 1699 cm^{-1} with small features on the high-frequency side. These transformations are interpreted as the formation of hydrogen bonds between some carbonyl ketones and water molecules; the band at 1671 cm^{-1} (fig. 10K) would indicate when these bonds are the strongest and most abundant, and the subsequent spectra indicate a decrease in the number of these bonds as the water molecules recede.

3.2.6. Drying the hydrated dry monolayer of Chl b

The monolayer of Chl *b* is dried in a vacuum chamber (0.1 Pa) at 65°C for 1.5 h and spectra N–Q (fig. 10) are taken at time intervals.

In the ester carbonyl region, there are some small modifications in the intensity pattern indicating that the ester groups are displaced; the same thing happens in the free ketone region.

In the aldehyde carbonyl region, there are many modifications leading to a strong component at 1662 cm^{-1} (fig. 10Q). This indicates that the associated aldehyde entity is decreased by the departure of the water giving some free aldehyde carbonyls.

The many modifications in the sample take place in the hydroxyl region where we see a strong absorption in the 3400 cm^{-1} range (fig. 10Q). This strong absorption has many small features which disappear in the next spectrum (fig. 10R). The appearance of this large band at 3400 cm^{-1} is interpreted as the departure of water of the second kind from the Mg atom of Chl *b*, and the trapping of these water molecules at the surface of the plate. The departure of these water molecules will affect the skeletal C=C, C=N bonds and we see that the bands of these groups in the 1610–1500 cm^{-1} range are modified (fig. 10Q) whereas before they had suffered few modifications.

4. Conclusion

The model that we have developed for a monolayer of Chl *a* at the air/water interface at a surface pressure of 20 mN m^{-1} consists of two sets of dimers of water situated on each side of a Chl *a* molecule linked to the water of the subphase by hydrogen bonds between the carbonyls and hydrogens of the water and forming an angle of 62.5°. One dimer is composed of two water molecules linked together by a hydrogen bond comparable to that observed in the gas phase and linked to one Chl *a* by a weak coordinate bond between the oxygen of one water molecule and the Mg atom on one side, and by weak hydrogen bonds between the hydrogens of the other water molecule and the π-electrons of another Chl *a* molecule. These water molecules constitute the water of the first kind, and are readily displaced in a competing situation. The other dimer is composed of two water molecules linked together by a hydrogen bond of the liquid-water type and is situated between the Mg atom and the water surface. The oxygen of one water molecule is strongly coordinate bonded to the Mg atom. This water molecule constitutes the water of the second kind and will be removed only under drastic conditions. The other water molecule is hydrogen bonded to the oxygen of the water at the surface of the trough. When deposited on a hydrophilic MIR infrared plate, the monolayer is transferred almost unaltered. The water of the first kind will rapidly disappear, and this will modify the monolayer. The infrared spectra which show a great number of peaks in the carbonyl region are sensitive to these modifications as shown by the continually modified absorption pattern observed with time in repetitive scans. When the water of the first kind has disappeared, that of the third kind will be next to follow, perturbing further the monolayer. Drying the monolayer under moderate conditions will remove most of the water of the third kind but not all water of the second kind. The departure of these water molecules will result in the collapse of the monolayer arrangement that a moist atmosphere will not restore but will affect individual functional groups dependent on their availability.

Comparison of the infrared spectra of a freshly prepared monolayer of Chl *a* with the resonance Raman work of Lutz [13] on intact chloroplasts whose photosensitive component is Chl *a* has revealed that the number of components and their positions are almost the same in both cases, differing only in intensity which could be due to the difference in temperatures of the samples. The interpretation of these results is that the association of the Chl *a* molecules with dimers of water, in a fashion similar to the aggregation of Chl *a* in monolayers, could play an important and essential role in the chloroplasts.

The model developed for a monolayer of Chl *b* at the air/water interface is essentially the same as for Chl *a*, except that the presence of an additional aldehyde group will modify slightly the aggregation state. The water of the first kind still consists of a water dimer linked together by a hydrogen bond and linked to the Mg atom by a coordinate bond on one end, and to the oxygen of the aldehyde carbonyl on the other end by a hydrogen bond with one hydrogen of the water dimer. This hydrogen bond is slightly stronger than the comparable bond in Chl *a* and because of the cooperative effort will produce a slightly increased pull on Chl *b*, resulting in an increased angle of tilt of the porphyrin plane to 66°. The distance between the central Mg atom and the surface of the subphase will be slightly increased, resulting in slightly reduced hydrogen bonding between water of the third kind and water at the surface. When the monolayer is transferred onto a MIR infrared plate, the spectrum will show some additional components due to the presence of the additional carbonyl group. These components are modified as the water of the first and third kinds leaves the surface. The original spectrum is not restored after putting the sample in a moist atmosphere, indicating a nonreversible reorganization and further alteration as the introduced water molecules form some bonds with the available groups.

When buildup of a multilayer of either Chl *a* or Chl *b* occurs, the water of the first kind will be displaced and a coordinate bond made between the ketone carbonyl (alternatively, between the aldehyde carbonyl if it is Chl *b*) and the Mg atom, resulting in a polymeric structure held in place by the undisplaced dimer of water composed of water of the second and third kinds. The water of the second kind is coordinate bonded to the Mg atom, and the water of the third kind is hydrogen bonded to the π-electron network in the case of Chl *a* or available carbonyls in the case of Chl *b*. As the water of the third kind departs from the multilayer, the buildup collapses, resulting in many polymeric breakups. The introduction of water molecules into the collapsed sample will not restore the original organization but will produce complexes of a nature different from those in a freshly made multilayer of chlorophyll.

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References

1 R.M. Leblanc and C. Chapados, Biophys. Chem. 6 (1977) 77.
2 C. Chapados and R.M. Leblanc, Chem. Phys. Lett. 49 (1977) 180.
3 C. Chapados, D. Germain and R.M. Leblanc, Biophys. Chem. 12 (1980) 189.
4 C. Chapados, D. Germain and R.M. Leblanc, Can. J. Chem. 59 (1981) 2402.
5 J.J. Katz and K. Ballschmitter, Angew. Chem. Int. Ed. 7 (1968) 286.
6 K. Ballschmitter and J.J. Katz, Nature 220 (1968) 1231.
7 K. Ballschmitter and J.J. Katz, J. Am. Chem. Soc. 91 (1969) 2661.
8 K. Ballschmitter and J.J. Katz, Biochim. Biophys. Acta 256 (1972) 307.
9 G. Sherman and S.F. Wang, Nature 212 (1966) 588.
10 G. Sherman and S.F. Wang, Photochem. Photobiol. 6 (1967) 239.
11 M. Lutz, C.R. Acad. Sci. Paris 275B (1972) 497.
12 M. Lutz, J. Raman Spectrosc. 2 (1974) 497.
13 M. Lutz, Biochim. Biophys. Acta 460 (1977) 408.
14 M. Lutz and J. Breton, Biochem. Biophys. Res. Commun. 35 (1973) 413.
15 M. Lutz, J.S. Brown and R. Rémy, Ciba Found. Symp. 61 (1979) 105.
16 G.L. Gaines, Jr., Thin Solid Films 68 (1980) 1.
17 S.M. de B. Costa, J.R. Froines, J.M. Harris, R.M. Leblanc, B.H. Orger and G. Porter, Proc. R. Soc. Lond. A326 (1972) 503.
18 R.M. Leblanc, G. Galinier, A. Tessier and L. Lemieux, Can. J. Chem. 52 (1974) 3723.
19 H.H. Strain and W.A. Svec, In: The chlorophylls, eds. L.P. Vernon and G.R. Seely (Academic Press, New York, 1966) ch. 2.

20 A.D. Buckingham, Proc. R. Soc. Lond. A248 (1958) 169.
21 V. Bekarek, M. Kaplanova, J. Socha, Stud. Biophys. Berl. Band 77 (1979) 21.
22 F.H. Stillinger, Science 209 (1980) 451.
23 T.R. Dyke, M.M. Mack and J.S. Muenter, J. Chem. Phys. 66 (1977) 498.
24 C. Kratky and J.D. Dunitz, J. Mol. Biol. 113 (1977) 431.