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AGGREGATION OF CHLOROPHYLLS IN MONOLAYERS.

II. Chlorophyll–dioxane interaction*‡

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The interaction of dioxane vapor with monolayer and multilayers of chlorophyll has been studied using electronic and infrared spectroscopies. Our results indicate the formation of a complex implying the oxygene of the dioxane molecules with the magnesiums of adjacent chlorophyll molecules. These results are consistent with the molecular orbital calculations, using the "free electron network" method done by Le Brech, Leblanc and Antippa [Chem. Phys. Letters 26 (1974) 37–44].

1. Introduction

A procedure to obtain a crystalline sample of a chlorophyll–lipoprotein complex was reported by Takashima [1]. The UV–visible absorption spectrum of this complex dissolved in a picoline/water mixture was given, however, no absorption spectrum was mentioned for the crystal. Krasnovskii et al. prepared the crystalline complex; the absorption spectrum of the crystal gave a peak at 690 compared to 670 nm when the crystal was dissolved in a picoline aqueous solution.

In the procedure used by Takashima [1] to get a crystalline sample of chlorophyll–lipoprotein, the mixture water/picoline/*p*-dioxane was used to precipitate the green chlorophyll–lipoprotein crystal. The nature of the crystalline formation suggests either a different association between lipoprotein and chlorophyll in the solid state compared to the complex in solution, or an association between *p*-dioxane (dioxane) and chlorophyll in the crystal. Krasnovskii et al. [2] provided evidence for the association of dioxane and chlorophyll which did not depend on the presence of protein. Electron diffraction and electron microscope studies of a solid film of chlorophyll *a* cast from a dioxane solution showed acicular crystals, however films cast either from an ether or from an ethanol solutions were amorphous [3].

Elucidation of the nature of the chlorophyll–dioxane association was attempted by Love and Bannister [4] through studies of colloidal chlorophyll in aqueous dioxane. From the differents salts of chlorophyll or pheophytin used, only Zn pheophorbide appears to form a dioxane colloid. The need for Mg or Zn suggests that dioxane is coordinated with the central metal. This hypothesis was confirmed by infrared studies; the incorporation of dioxane breaks any association with the C_9 carbonyls of the chlorophylls [5].

Recently, a molecular orbital calculation based on a chlorophyll–dioxane "interaction model" was made [6]. As seen in fig. 1, the important features of the "interaction model" are *that dioxane is bonded to the central magnesium atom, that it interacts with aggregates rather than single molecules of chlorophyll, and that the central magnesium atom has a coordination number of SIX in this complexed state.*

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* Part I is referred to: The absorption spectrum of chlorophyll *a* and the chlorophyll/dioxane and chlorophyll/benzoquinone interactions [J. Le Brech, R.M. Leblanc and A.F. Antippa, Chem. Phys. Letters 26 (1974) 37–44].

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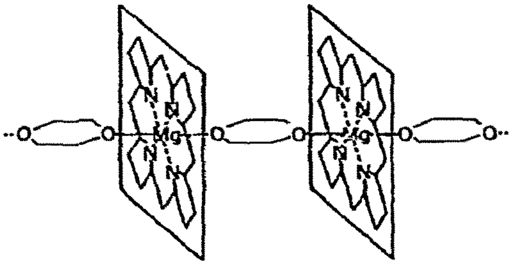

Fig. 1. Chlorophyll *a*–*p*-dioxane interaction: model of the complex.

In the present paper, we examine the chlorophyll–dioxane interaction in a monolayer assemblies using electronic and infrared spectroscopies.

2. Experimental

The materials, the preparation of slides, the deposition of monolayers, the float trough and the Langmuir film balance apparatus have previously been described in detail [7].

2.1. Electronic spectra

Before depositing the chlorophyll *a* monolayers, the glass slides were coated with five layers of cadmium arachidate using the float trough apparatus. With the underlying layers on, each dipping of the glass slide through the water surface covered with chlorophyll *a* monolayer yielded two layers on each side of the slide. The Langmuir film balance was used to monitor the deposition of chlorophyll *a* on to slide. With the present method of preparation, the chlorophyll layers are always situated at a lipid/air interface. Four to six slides were prepared before flushing the nitrogen/dioxane gaseous mixture on these slides.

The absorption measurements were carried out on a Perkin-Elmer 124 spectrophotometer fitted with a HTV R136 photomultiplier. The fluorescence measurements were made with an Aminco-Keirs spectrofluorimeter incorporating a HTV R213 photomultiplier. Details of the slide(s) arrangement in the light path have been given in ref. [7].

2.2. Infrared spectra

No cadmium arachidate base was used for the infrared spectra. Using the Langmuir film balance, forty layers (multilayers) of chlorophyll *a* or *b* on both sides of a plate were needed for a 40–50% absorption for the strongest carbonyl band.

The multilayers were dried at 75°C under a vacuum of 10^{-3} torr. The anhydrous chlorophyll was put in a dioxane vapor atmosphere (40 torr) for several hours and the spectra were taken at different time intervals to observe the chlorophyll multilayers–dioxane interaction. To get rid of all the dioxane, the multilayers were heated again at 75°C under a vacuum of 10^{-3} torr.

The infrared spectra were taken on a Unican 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. The internal reflection sampling technique was used since the absorption of the multilayers was far too low. Germanium and AgBr plates gave good results. The cleaning procedure of the plate was particularly important since it affects the deposition ratio (d.r. = area monolayer removed/area of slide dipped) of the monolayer and subsequently, the quality of the spectra. The procedure which gave the best results was the one developed by Takenaka et al. [8].

3. Results and discussion

3.1. Electronic spectra

Electronic spectra of chlorophyll *a* in monolayers and multilayers have been examined at the air/solid interface [7].

There is presumably a distribution of aggregate sizes of chlorophyll *a* at the air/lipid interface. The chlorophyll *a* aggregates are formed at the air/aqueous interface during the evaporation of the spreading solvent and the compression of the layer. Dipping a glass slide coated with five layers of cadmium arachidate, with its long axis perpendicular to the water surface, would make this direction a "preferred" one for the monolayer deposit. The stacks of chlorophyll *a* molecules attached together with their porphyrin

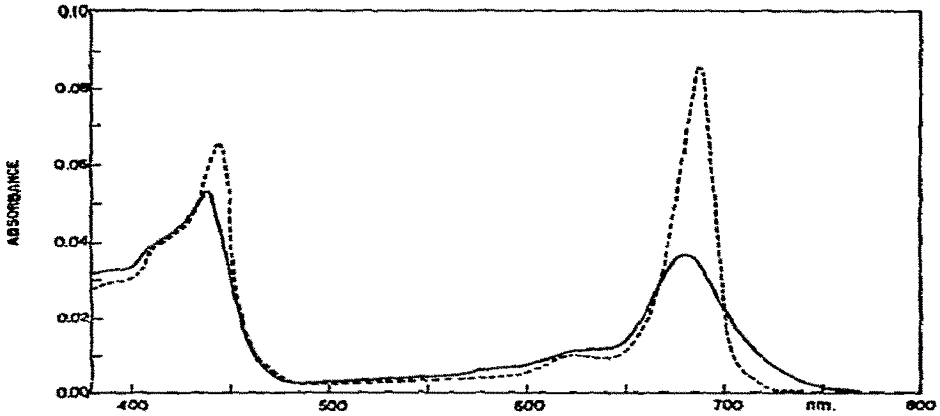

Fig. 2. Absorption spectra of chlorophyll *a* double layers deposited at a constant surface pressure (20 m N m^{-1}) at a lipid/air interface: initial spectrum (——); after exposure (180 min) to dioxane vapor at 20°C (– – –).

planes more or less parallel to each other could be expected to deposit on to the hydrophobic slide with an orientation parallel to the dipping direction. Fluorescence polarization measurements of chlorophyll *a* monolayers have shown an orientation effect of chlorophyll *a* molecules in the monomolecular state [9].

The effect of dioxane vapor on the absorption spectra of chlorophyll *a* double layers is shown in fig. 2. The presence of dioxane in the chlorophyll *a* double layers produce a bathochromic shift of 8 nm in the absorption spectra. The relative absorbance at the red band is also much increased. Leaving the slide for a few hours in the dark and in the nitrogen atmosphere, we recover the initial spectrum with the same absorbance and λ_{max} at the Soret and at the red bands. Repeating the experiments two or three times does not change the absorption properties of the chlorophyll *a* double layers.

We believe that the effect of dioxane might be to reorient the chlorophyll *a* molecules to give the interaction model proposed in fig. 1. Some experimental evidences are in favor of such a model:

(i) The main absorption bands of chlorophyll *a* in a dioxane solution are at 662.0 and 433.1 nm, and the corresponding bands of chlorophyll *a* double layers in dioxane vapor are at 688 and 446 nm. The interaction of dioxane with chlorophyll *a* double layers must be different compared to the one in a dioxane solution.

(ii) Replacing dioxane vapor by piperidine does not affect the absorption spectra, however piperazine vapor produces the same shift as with dioxane. It seems that we need an electron donating atom at both ends of the molecule to obtain an effect similar to the dioxane. So, morpholine vapor produces a shift identical to the dioxane vapor but diethyl ether does not give any shift.

(iii) By diluting the chlorophyll *a* multilayers with an inert diluent such as cadmium arachidate or the mixture barium oleate/barium stearate (1:1), and when exposing the multilayers to dioxane vapor, we observe that the intensity of the absorption of the red band is less increased. The absorbance in the initial spectra of the chlorophyll *a*/diluent multilayers was the same as the absorbance of the chlorophyll *a* double layers initial spectra. The dilution of chlorophyll *a* in the multilayers system decreases the number of dimers or higher aggregate species complexing with the dioxane.

(iv) Within the sensitivity of the apparatus we detected no change in the fluorescence spectra ($\lambda_{emiss.}$ = 680 nm) of chlorophyll *a* double layers when exposed to dioxane vapor. Since the fluorescence for the chlorophyll *a* monomer has been observed [7], we suggest that the dioxane complexes with dimers, trimers ... or oligomers of chlorophyll *a*.

(v) The absorption spectra of a transparent solid solution of chlorophyll *a* in a lecithin matrix resemble the spectra in an ether solution [10]. When the dioxane vapor is flushed on a solid film solution of

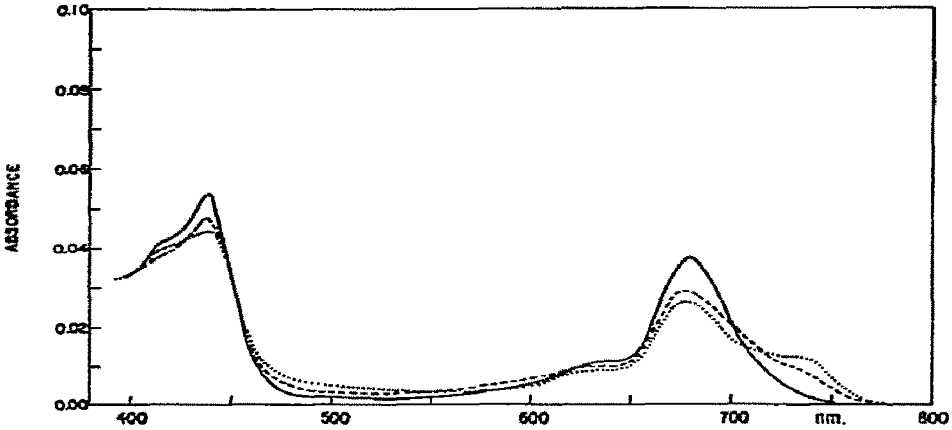

Fig. 3. Absorption spectra of chlorophyll *a* double layers deposited at a constant surface pressure (20 m N m^{-1}) at a lipid/air interface: initial spectrum (——); after exposure to water vapor at 20°C, 18 hrs (– – –), 90 hrs (···).

chlorophyll *a* in lecithin, no change is observed on the absorption spectrum of the film. This result gives further evidence that dioxane does not complex with chlorophyll *a* monomer.

(vi) The effect of water vapor on chlorophyll *a* double layers is shown in fig. 3. There is apparition of a band at 742 nm after 90 hours exposure. When leaving the slide in the dark and in the nitrogen atmosphere, we do not recover the initial spectrum. Probably, the chlorophyll *a* molecules in a monomolecular state do not have the orientation necessary to get easily the 742 chlorophyll *a*–water interaction. We can see that the chlorophyll–water complex has a different nature than the chlorophyll *a*–dioxane complex.

All these observations agree with the interaction model proposed in fig. 1. We attribute the increase in absorption of the complex chlorophyll–dioxane to a splitting of large aggregates or oligomers to smaller ones.

3.2. *Infrared spectra*

Infrared spectra of chlorophylls in solution and in solid state have been reviewed [11], and the bands in the carbonyl region assigned. An IR study of the chlorophylls in different solvents has been made [12] for determining the structure of the dimers and oligomers species and the state of aggregation has been confirmed by molecular weight measurements [13].

Visible and infrared spectra of dry chlorophyll have been recorded by Ballschmiter et al. [14] hereafter called I. We refer to this article for previous solid works and for assignment of the bands in the carbonyl region. In that article, the effect of water on the spectra has been studied and a correlation was done between visible spectra and IR spectra which serve to determine the state of aggregation.

Although the effect of water is important in the multilayers of chlorophyll we will not discuss it since we use a dried sample, and we will leave the water effect for a further work (C. Chapados and R.M. Leblanc, unpublished).

The spectra of 40 layers of chlorophyll *a* and chlorophyll *b* are given in figs. 4 and 5 respectively. The spectrum A is the background spectrum of the ATR plate in the region 2000–1500 cm^{-1}. In the 4000–2500 cm^{-1} region the background has been subtracted from the spectra in order to have the true absorption pattern in the hydroxyl region. The spectrum B is taken after drying the sample: for chlorophyll *a* the drying is done at 75°C under a vacuum of 10^{-3} torr for one hour; for chlorophyll *b* the drying is done in the sample compartment of the spectrophotometer for 3 hours. Spectrum C is taken after exposing the multilayers of chlorophyll to the dioxane vapor: 14 hours for chlorophyll *a*, 12 hours for chlorophyll *b*. Spectra D and E are taken at time intervals after spectrum C. Spectrum F is taken after heating the sample at 75°C under a vacuum of 10^{-3} torr for

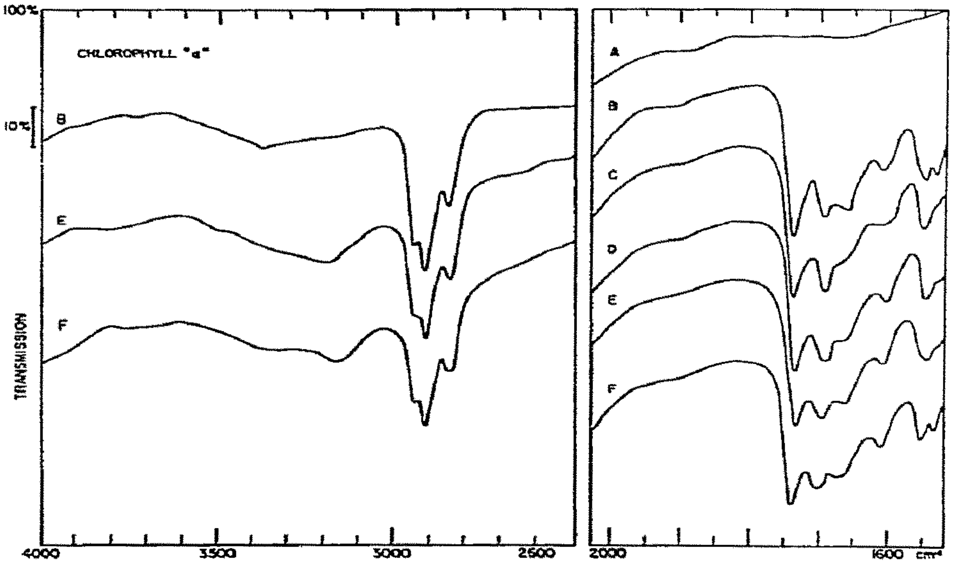

Fig. 4. Infrared spectra of 40 layers (multilayers) of chlorophyll *a*. A, background; B, anhydrous multilayers; C, after 14 hrs in dioxane vapor; D, 15 min later; E, 94 min later; F, after heating at 75°C under a vacuum of 10^{-3} torr for 90 min.

90 min in order to get rid of all the dioxane. The data are tabulated in table 1 for chlorophyll *a* and in table 2 for chlorophyll *b* with the one obtained by 1 for the solid film. After the dioxane vapor effect, we

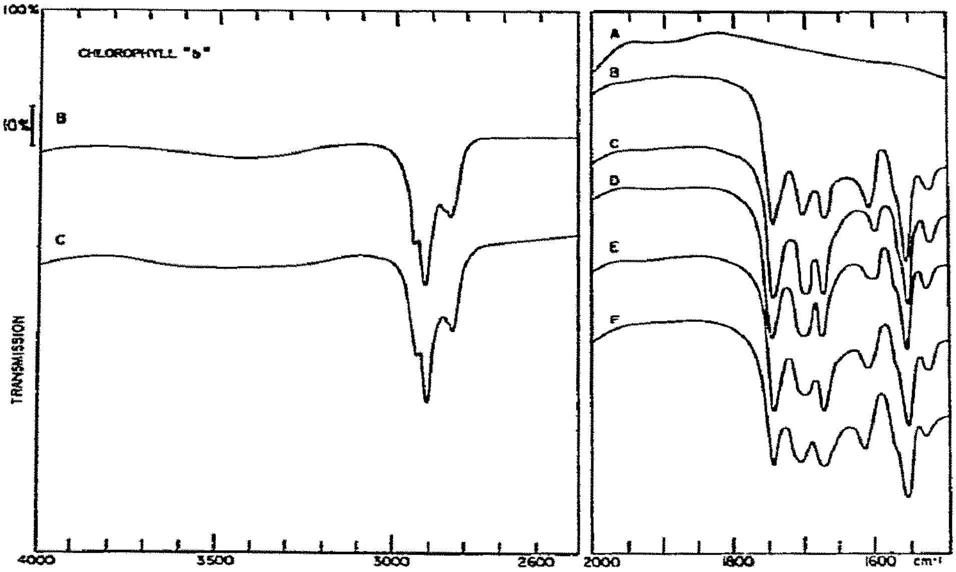

Fig. 5. Infrared spectra of 40 layers (multilayers) of chlorophyll *b*. A, background; B, dry multilayers; C, after 12 hrs in dioxane vapor; D, 20 min later; E, 90 min later; F, after heating at 75°C under a vacuum of 10^{-3} torr for 150 min.

Table 1
Frequencies (cm^{-1}) of multilayers of chlorophyll *a*

No.	Spectrum	Description	Ester C=O	Free keto C=O	Coordinated keto C=O···Mg	Skeletal C=C C=N	Skeletal
1	*	Anhydrous solid film	1736	1695	1655	1610	1550 1530
2	B	Anhydrous multilayers	1737	1692	1658	1609	1550 1535
3	C	After 14 hours in dioxane vapor	1738	1691	1660(sh)†	1610	1548 1534(sh)†
4	D	After 15 min	1739	1695	1666	1608	1551 1532(sh)
5	E	After 94 min	1739	1699	1665	1611	1549 1529(sh)
6	F	After heating under vacuum for 90 min	1737	1698	1666	1608	1548 1528

* K. Ballschmitter et al. [14]. Solid film cast from CCl_4 solution.
† sh: shoulder.

detected no band that could be attributed to the dioxane.

3.2.1. *Multilayers of chlorophyll a*

Comparing the spectrum of an anhydrous multilayers of chlorophyll *a*, fig. 4B, with the one obtained by I for the anhydrous solid film, we see that the position (table 1) of the C=O ester band, the skeletal C=C, C=N vibration band and the 1550 cm^{-1} branch of the doublet are the same. The other branch of the doublet is at a higher frequency in the multilayers than in the solid film. The intensity pattern of these bands are also the same. Compared to the intensity of the C=O ester band, the intensity of the free C=O ketone band of the multilayers is approximately the same as the one obtained for the solid film. However the position in the multilayers is at a slightly lower frequency than the one obtained for the solid film, 1692 cm^{-1} compared to 1695 cm^{-1}. In solutions of chlorophyll *a* in CCl_4 [11–13], tetrahydrofuran [11], *n*-butylcyclohexane [12,13], hexane [13] and 1,2-dichloroethane [13] the position of the free C=O ketone band is respectively at 1695, 1696, 1704, 1704 and 1690 cm^{-1}. When the concentration in aliphatic solvent increases, the position of this band decreases indicating greater interaction of the free C=O ketone

Table 2
Frequencies (cm^{-1}) of multilayers of chlorophyll *b*

No.	Spectrum	Description	Ester C=O	Free keto C=O	Coordinated keto C=O···Mg Free aldehyde C=O	Coordinated aldehyde C=O···Mg Skeletal C=C, C=N	Skeletal
1	*	Anhydrous solid film	1736	1700	1663	1606	1550 ?
2	B	Dry multilayers	1739	1698	1668	1605	1552 1520
3	C	After 12 hours in dioxane vapor	1740	1694	1669	1597	1549 1519
4	D	20 min later	1740	1695	1669	1597	1549 1521
5	E	90 min later	1739	1695	1669	1605	1549 1521
6	F	After heating under vacuum of 10^{-3} torr for 150 min	1739	1699	1668	1609	1549 1524

* K. Ballschmitter et al. [14]. Solid film cast from C_6H_6.

with the environment. So a position at 1692 cm^{-1} would indicate that the interaction of the free C=O ketone with adjacent chlorophyll molecules is greater in the multilayers than in the solid film and in the solutions, except for the solution in 1,2-dichloroethane where the low position at 1690 cm^{-1} is not understood [13]. This greater interaction of the free C=O ketone with the environment in the multilayers of chlorophyll *a* is understandable. In the multilayers the chlorophyll molecules are less free to move than in solution or even in the solid film, in the later the chlorophyll molecules can orient themselves before the solvent evaporates.

The position of the coordinated C=O ketone band is at 1658 cm^{-1}. In the solid film of I, the position is at 1655 cm^{-1} where the aggregations are considered mainly dimers. In a 0.0017 M solution of chlorophyll *a* in hexane [13] the position of this band is at 1657 cm^{-1} where the aggregations are mainly oligomers. Increasing the concentration of chlorophyll *a* in aliphatic solvent and consequently increasing the concentrations of oligomers causes a shift to higher frequencies of the coordinated ketone band. In the multilayers, the coordinated ketone band is broader than in the solid film and in solutions, so the aggregation structure of the multilayers consists of dimers, trimers and oligomers with the later being the predominant species due to the high position of the coordinated ketone band. The relative intensity of this band is weaker in the multilayers than in the solid film, so there is less coordinated C=O ketone in the multilayers than in the solid film.

The influence of dioxane vapor on the multilayers of chlorophyll *a* is illustrated in fig. 1C. The intensity of the free ketone band at 1692 cm^{-1} increases while the coordinated ketone band at 1658 cm^{-1} disappears almost completely. The skeletal C=C, C=N band at 1609 cm^{-1} decreases in intensity and broadens. One branch of the doublet (1552–1535 cm^{-1}) disappears almost completely leaving a shoulder. There is little shift in the position of the bands (table 1). With aging (fig. 4D and E) there is a gradual increase in the coordinated ketone band and a corresponding decrease in the free ketone band. Upon heating (fig. 4F) this phenomenon is accentuated and the doublet is restored (1548–1528 cm^{-1}).

We explain these phenomena by the interaction of the dioxane molecules with the chlorophyll molecules. When the dioxane is in contact with the chlorophyll, the coordinate C=O···Mg bond is ruptured which is illustrated by the almost complete disappearance of the coordinated ketone band. This can be done only by forming a coordinated complex: Mg···OC_4H_8O···Mg, so the Mg coordinates with the oxygen of the dioxane and is therefore no more available for coordination with C=O carbonyl of adjacent chlorophyll molecules. The interaction of the dioxane with the chlorophyll is further illustrated by the decrease in intensity of the skeletal bands at 1609 and 1535 cm^{-1}.

As the dioxane molecules leave the multilayers: (a) there is a restoration of the coordinated ketone band, (b) the former π electron network shown on the spectra by the increase of the coordinated ketone band at 1608 cm^{-1}, (c) the restoration of the 1528 cm^{-1} branch of the doublet. The high position of the free C=O ketone band at 1698 cm^{-1} (fig. 4F and table 1) would indicate that most of the free ketones are essentially free of interactions with the environment but since this band is broader than in the previous spectra, some of the free ketones do interact with their environment. The coordinated C=O···Mg ketone band is broad indicating a wide varieties of species from dimers to oligomers but since the band position is at 1666 cm^{-1}, the predominant species would be oligomers.

The small difference between the spectrum taken before introducing the dioxane (fig. 4B) and after removing it (fig. 4F) is caused by the perturbation of the multilayers by the dioxane.

3.2.2. Multilayers of chlorophyll b

Comparing the spectrum of the dry multilayers of chlorophyll *b* (fig. 5B, table 2) with the anhydrous solid film of I, we notice that the relative intensity of the 1668 cm^{-1} band is greater in the multilayers than the 1663 cm^{-1} band of the dry film. So we have to consider that this band contains much more coordinated ketones in the multilayers than in the dry film. The small displacement in the frequencies can be attributed to a weaker coordinated bond caused by the organisation of the multilayers. The band at 1605 cm^{-1} attributed to coordinated C=O aldehyde and to skeletal vibrations is more intense in the multilayers than in the solid film. This is caused by more coordinated C=O aldehyde in the former than in the latter. These results suggest that there is more aggregation in the multilayers than in the solid film. Since a trimer has been proposed by I for the solid film, we propose for the multilayers of chlorophyll *b* an aggregation state consisting predominantly of oligomers.

Exposure of the multilayers to dioxane vapor (fig. 5C) causes the sharpening, the increase in intensity and the displacement to lower frequency from 1698 to 1694 cm^{-1} of the free ketone band, the sharpening and the increase in intensity of the 1668 cm^{-1} band, the decrease in intensity and the displacement to lower frequency of the 1605 cm^{-1} band (table 2). The other bands are little affected by the dioxane vapor.

These modifications are consistent with the model proposed for chlorophyll *a* and which consisted of a coordinate bond formed between the oxygen of the dioxane and the Mg of the adjacent chlorophyll molecules. This interaction breaks most of the C=O···Mg coordination of adjacent chlorophyll molecules.

The increase in intensity of the free ketone band is caused by the new available free ketone. The slight decrease in frequency is explained by hydrogen bonding between the free C=O ketone with either the dioxane molecule or the adjacent chlorophyll molecules. The sharpening of the 1666 cm^{-1} band (fig. 5C) is explained by the fact that most of the coordinated C=O ketone vibrations has disappeared in the chlorophyll–dioxane interaction, and the band now contains mainly the free C=O aldehyde. The increase in intensity of this band is due to the new available free C=O aldehyde since most of the coordinated C=O aldehyde vibrations has disappeared. This disappearance is evident on the 1605 cm^{-1} band which is decreased in intensity and displaced to 1597 cm^{-1}. The remaining band contains C=C, C=N skeletal vibrations.

Upon aging (fig. 5D and E) and after heating (fig. 5F), the bands resume their former positions and their former intensity. This is consistent with the reformation of the C=O···Mg coordinated bond as the dioxane molecules leave the multilayers.

In the hydroxyl region, the spectra of dry multilayers of chlorophyll *a* (fig. 4) and of chlorophyll *b* (fig. 5) show a large, structureless, low intensity band. The introduction of dioxane (figs. 4E and 5C) produces a small increase in intensity and some displacement of the bands centers. Removal of the dioxane (fig. 4F) causes some small changes in the absorption pattern. Presently these modifications are not entirely understood and some efforts are being made to determine the origin of the band and the modifications caused by the introduction of dioxane molecules in the multilayers of the chlorophylls.

4. Conclusion

When the dioxane vapor interacts with chlorophyll *a* in a monomolecular state, we observe a band situated at 688 nm and one at 444 nm in the absorption spectrum. The bands are red shifted by 8 nm from the absorption spectrum of chlorophyll *a* double layers or multilayers. The 688 nm band width is much narrower than the one without dioxane. A complete reversibility is observed in the absorption spectrum. We attribute these changes to the formation of a complex between chlorophyll and dioxane. The nature of this complex is examined by infrared studies of chlorophyll multilayers. It is shown that the Mg of the chlorophyll *a* or *b* interacts with the oxygen of the dioxane molecule breaking most of the coordinate bands between adjacent chlorophyll molecules. This is seen on the IR spectra by the almost complete disappearance of the coordinated ketone band. Our data agree with the "interaction model" proposed by Le Brech et al. [6] for the chlorophyll–dioxane complex.

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