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Preparation of Multilayers Containing Chlorophyll *a* and/or Phosphatidylcholine and Chemical Stability of Chlorophyll *a* Molecules in the Multilayers

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Summary. The Langmuir-Blodgett films consisting of mixed monolayers containing chlorophyll *a* (Chl-*a*) and/or phosphatidylcholine with various molar ratios could be well prepared on glass plates previously covered with three monolayers of cadmium eicosanate. Thin-layer and high-performance liquid chromatographic examinations revealed that the photostability of Chl-*a* molecules in various media is in the following order: the mixed mono- or multilayers $>$ pure Chl-*a* mono- or multilayers $\gg$ organic solvents.

It has generally been recognized that photosynthesis is carried out in the chloroplast where chlorophyll (Chl) molecules and all other photosynthetic materials are incorporated into highly organized lamellar structures. The concept of Chl aggregate with the layered structure has induced numerous studies on the Langmuir-Blodgett films of Chl as a biomimetic membrane system [11]. The aggregation of Chl molecules in vitro should be quite different from that of Chl molecules in vivo. Nevertheless, a detailed knowledge of the two-dimensionally packed aggregate forms in vitro would be neccessary to gain insight into the stability of Chl molecules and also to elucidate the relationship between the state and role of Chl in photosynthesis.

The present series of work [1, 2, 7, 8] was designated to investigate the properties of Chl in closely packed layers (Langmuir-Blodgett films). Recently, we have re-examined and have established methods for the preparation of chlorophyll *a* (Chl-*a*) monolayers [7] and multilayers [8] and have also developed a method for the preparation of phosphatidylcholine (PC) multilayers [5]. In this article, the chemical stability of Chl in multilayers as well as the method for the preparation of the mixed multilayers consisting of Chl-*a* and PC monolayers will be reported.

Materials and Methods

All the experiments were performed at 20±2 °C in total darkness or under dim green light unless otherwise stated.

Chl-*a* was carefully prepared from fresh spinach leaves according to the method of Iriyama, Shiraki and Yoshiura [9] or from *Porphyra yezoensis* according to the method of Iriyama [6]. The molar extinction coefficients of Comar and Zscheile [4] were used in the determination of the concentrations of Chl-*a*. The purity and chemical stability of Chl-*a* were examined by thin-layer chromatography [16] and by high-performance liquid chromatography [10, 19]. Thin-layer and high-performance liquid chromatographic analyses revealed that Chl-*a* preparations used did not contain any leaf yellow pigments, chlorophyll *b* or degradation products of Chl. Absorption spectra of the Chl-*a* preparations dissolved in diethyl ether did not show any significant differences in comparison with the literature [18] within experimental error; the ratio of absorbance of the Soret peak at 427.0 (±1.0) nm to the red peak at 660 (±0.5) nm was 1.30 (±0.01). The purity of Chl-*a* preparations was 99.0–100% on dry wt basis. Pheophytin *a* (Pheo-*a*), and chlorophyll *a′* (Chl-*a′*) used in thin-layer chromatographic analysis as standard materials were prepared according to the procedures previously described [16]. Phosphatidylcholine was prepared from egg yolk according to the method of Shimojo and Ohnishi [15]. The preparation showed a single spot of PC on a thin-layer chromatogram (adsorbent, Kieselgel G; developing solvent system, chloroform/methanol/water, 14:6:1, vol/vol/vol). The phosphorous content and the purity of PC were 3.81 and 99.6%, respectively.

All the spectrophotometric measurements were carried out in a Carry 14 R automatic spectrophotometer.

The surface pressure-area characteristics were measured using Wilhelmy's plate method with a precision of $\pm 0.025\,\mathrm{mN\,m^{-1}}$ for surface pressures. Measurements were repeated more than three times and the average value was used. A glass trough coated with highly purified paraffin was placed in a covered thermostated space which permitted operations in a controlled atmosphere and light conditions. The thin films containing Chl-*a* and/or PC were spread under dim green light on the aqueous subphase (10^{-3} M pH 8 phosphate buffer) at 20 °C in nitrogen atmosphere using 10^{-3}–10^{-4} M of Chl-*a* and/or PC in wet benzene saturated with 10^{-3} M phosphate buffer as spreading solution [7]. All the mixed monolayers prepared and studied were in fairly condensed states at least in surface pressure range from $7\,\mathrm{mN\,m^{-1}}$ to $22\,\mathrm{mN\,m^{-1}}$.

Multilayers containing Chl-*a* and/or PC were prepared according to the procedures described elsewhere [8]. Deposition ratios were always measured to assure perfect deposition of monolayers

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into a multilayer structure. The deposition ratio is defined as $Ar/Ad \cdot n$, where n is the number of monolayers piled on a solid substrate, Ad is the surface area of substrate covered with a multilayer (or monolayer), and Ar is the total area of monolayer on water that has been taken up on a solid substrate. When the deposition is perfect, the deposition ratio should be unity. An apparatus for the preparation of Langmuir-Blodgett films developed by Kuhn, Möbius and Bücher [12] was employed in this study. All the mixed monolayers prepared on aqueous subphase could be well deposited on glass plates previously coated with three monolayers of cadmium eicosanate prepared according to the method of Kuhn *et al.* [12], although we have not been very successful in depositing Chl-*a* monolayers directly on glass plate. 1.0 × 4.0 cm glass plates were treated for 6 hr at room temperature with an ethanol solution containing 5% distilled water which was saturated with KOH, then rinsed well with distilled water, and dried in an oven. The glass plates thus treated were used as solid substrate for monolayer deposition. It was confirmed spectroscopically and chromatographically that Chl-*a* molecules in the freshly prepared multilayers did not change chemically.

The multilayers consisiting of the mixed monolayers containing Chl-*a* and PC were illuminated with 500 W xenon lamp at 50 cm for 1-min time intervals through water filter to cut off heat. When neccessary, the irradiated samples were dipped into diethyl ether to dissolve the multilayers, and the solutions were analyzed by thin-layer chromatography [16] and high-performance liquid chromatography [10, 19] as well as by spectrophotometry.

Results and Discussion

The surface pressure-area characteristics of Chl-*a* and PC monolayers on the aqueous subphase (10^{-3} M pH 8 phosphate buffer) are shown in Fig. 1. The surface pressure-area characteristics of Chl-*a* monolayer were in good agreement with the values by Bellamy, Gaines and Tweet [3] within experimental error. Chl-*a* and PC monolayers, under the present conditions, reached a collapsing point at 22.0 mN m^{-1} and 43.0 mN m^{-1}, respectively. These pressures are designated as the collapsing pressures ($F_{\max}$). The limiting area per molecules ($A_{F=0}$) may be defined as the area occupied by one molecule in the monolayer prepared on the aqueous subphase when the pressure is extrapolated to zero. Figure 2 shows a typical surface pressure-area characteristics of the mixed monolayer containing Chl-*a* and PC, which was spread from a 1:2 mixture dissolved in the wet benzene. In this case, $F_{\max}$ and the observed $A_{F=0}$ were 22.0 mN m^{-1} and 91.6 Å²/molecule, respectively. A list of the data for $F_{\max}$ and $A_{F=0}$ is presented in Table 1. $A_{F=0}$ and $A_{F=16.5}$ were calculated by the following equations:

$$A_{F=0} = Ma \cdot A_{F=0}(a) + Mp \cdot A_{F=0}(p) \quad (1)$$

$$A_{F=16.5} = Ma \cdot A_{F=16.5}(a) + Mp \cdot A_{F=16.5}(p) \quad (2)$$

where Ma and Mp are mole fractions of Chl-*a* and PC in monolayers, respectively. $A_{F=16.5}(a)$ and $A_{F=16.5}(p)$ are the corresponding values of area for Chl-*a* and PC in the monolayers at a surface pressure of 16.5 mN m^{-1} at which most of the following experiments were performed. With decreased molar ratios of Chl-*a* and PC, the $F_{\max}$ values of the monolayers became higher.

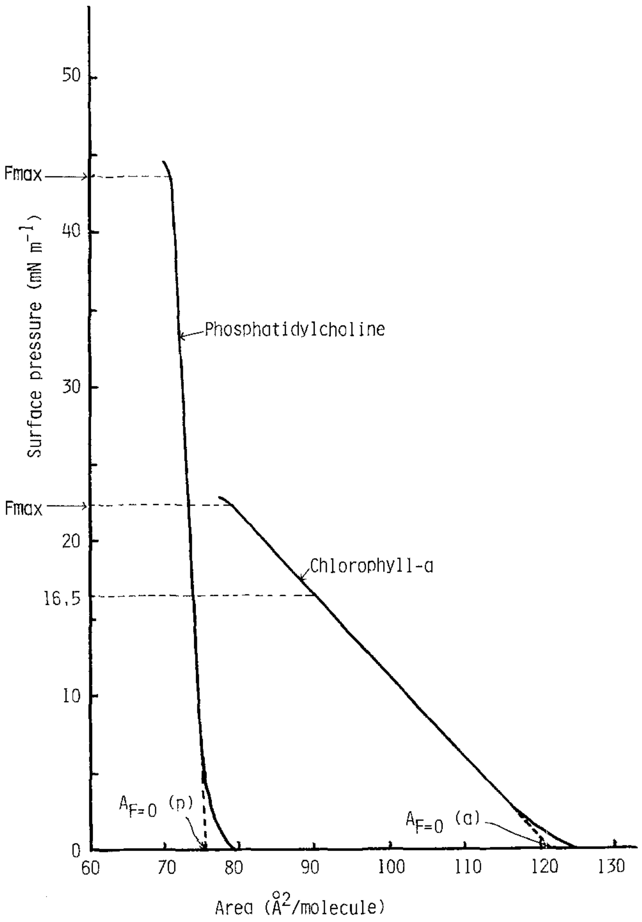

Fig. 1. Surface pressure-area characteristics of chlorophyll *a* and phosphatidylcholine monolayers on 10^{-3} M pH 8 phosphate buffer, N_2 atmosphere, 20 °C

Deposition ratios were determined for all the multilayers used in this study. Table 2 presents deposition ratios of forty layers of Chl-*a* and/or PC on the glass plates previously covered with three cadmium eicosanate monolayers at a surface pressure of the mother monolayers of 16.5 mN m^{-1}. Values nearly equal to unity were consistently obtained. The high values of deposition ratio (near unity) obtained throughout this experiment indicate that molecules constituting the mother monolayer were transferred onto the surface of the plates or on the top layer of the already deposited multilayers without changing the uniformity of distribution and the surface-density of the materials in the monolayers.

Absorption spectra of the multilayers prepared from monolayers containing different molar ratios of Chl-*a* to Pc were measured. Figure 3 shows absorp-

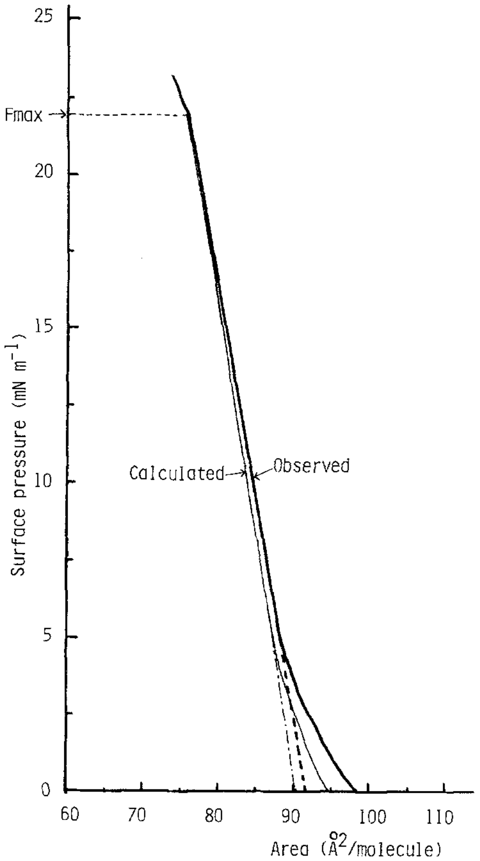

Fig. 2. Surface pressure-area characteristics of the mixed monolayer containing chlorophyll *a* and phosphatidylcholine on 10^{-3} M pH 8 phosphate buffer, N_2 atmosphere, 20 °C. The monolayer was spread from a 1:2 mixture. *See also* Tables 1 and 2

Table 1. Data from surface pressure-area characteristics of the mixed monolayers of Chl-*a* and PC with various molar ratios on 10^{-3} M pH 8 phosphate buffer at 20 °C

Chl-*a*/PC (molar ratio)	F_{max}[a] (mN m^{-1})	$A_{F=0}$[a] (A^2/molecule)		$A_{F=16.5}$[a] (A^2/molecule)	
		Calc.[b]	Obs.[c]	Calc.[b]	Obs.[c]
1: 0	22.0	–	121.0	–	90.0
1: 1	21.6	98.3	100.2	82.0	82.1
1: 2	22.0	90.7	91.6	79.3	79.5
1: 4	22.8	84.8	86.1	77.2	77.8
1: 6	23.1	82.1	83.8	76.5	76.7
1: 8	23.4	80.6	80.9	75.8	75.9
1:10	23.7	79.7	80.0	75.5	75.8
1:50	24.2	76.5	76.5	74.3	74.3
0: 1	43.0	–	75.6	–	74.0

[a] For explanations, *see* the text.
[b] Calculated values.
[c] Observed values.

Table 2. Deposition ratios in preparing the multilayers from the mixed monolayers of Chl-*a* and/or PC with various molar ratios onto glass plates previously coated with three cadmium eicosanate monolayers at 20 °C

Chl-*a*/PC (molar ratio)	Surface pressure (mN m^{-1})	Moving rate of glass plate (cm/min)	Deposition ratio
1: 0	16.5	0.5	0.99
	16.5	1.0	0.98
1: 1	16.5	0.5	0.97
	16.5	1.0	0.98
	16.5	5.0	0.78
	20.0	1.0	0.97
	20.0	0.1	0.98
1: 2	16.5	0.5	0.98
	16.5	1.0	0.97
	20.0	0.5	0.99
1: 4	10.0	0.5	0.91
	10.0	1.0	0.87
	16.5	1.0	0.98
1: 6	16.5	1.0	0.99
1: 8	16.5	1.0	0.98
1:10	16.5	1.0	0.96
		0.5	0.99
1:50	10.0	0.5	0.92
	10.0	1.0	0.89
	16.5	0.5	0.96
	16.5	1.0	0.94
	20.0	1.0	0.98
0: 1	16.5	1.0	0.94

tion spectra of multilayers as examples. Table 3 presents the peak position of the red and blue bands and the absorbance ratios (blue/red) along with the film concentrations. As expected, it seems that the positions of the red and blue bands depend on the molar ratios of Chl-*a* and PC in the monolayers. The absorption maxima shifted to longer wavelengths when the mole fractions of Chl-*a* in the monolayers were increased.

The pure Chl-*a* multilayer prepared at the surface pressure of the mother monolayer of 16.5 mN m^{-1} was irradiated by light from xenon lamp in air. Chl-*a* molecules in the multilayer were stable against irradiation for at least 2 hr. Thin-layer chromatographic analysis revealed that when the film irradiated for longer periods, Chl-*a* molecules in the film were converted to Pheo-*a*, oxidized Chl-*a*, and some unidentified degradation products of Chl-*a*. Figure 4 shows the thin-layer chromatogram of pigments in the irradiated film. One of the degradation products did not move on the thin-layer chromatogram and was not identified. The chemical reactions of Chl-*a* were very complicated to produce many unidentified photodegradation products of Chl-*a*. In addition, it has been found that the rate of the photodegradation of Chl-*a* in solutions was more rapid [17]. When the irradiation was performed in nitrogen atmosphere, the oxidized Chl-*a* was not formed in the multilayers. The chemical structure of the oxidized Chl-*a* still re-

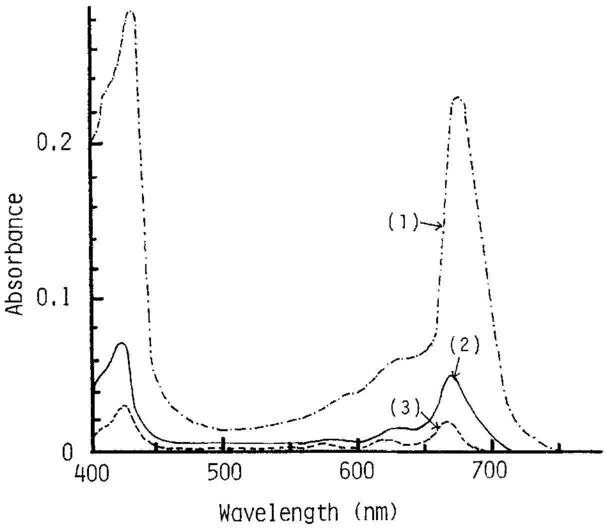

Fig. 3. Absorption spectra of several mixed multilayers of chlorophyll *a* and phosphatidylcholine. The multilayers (*1*), (*2*) and (*3*) were prepared from the mixed monolayers spread from 1:0, 1:4, and 1:10 mixtures at a surface pressure of 16.5 mN m^{-1} at 20 °C in a N_2 atmosphere. Twenty monolayers were deposited for each multilayer preparation. *See also* Table 3

Table 3. Some properties of the absorption spectra of the forty layers on the glass plates prepared from the mother monolayers of Chl-*a* and PC with various molar ratios at a surface pressure of 16.5 mN m^{-1} at 20 °C

Chl-*a*/PC (molar ratio)	Peak position in nm		Absorbance ratio blue/red
	Blue band	Red band	
1: 0	435	675	1.24
1: 1	435	675	1.24
1: 2	434	674	1.25
1: 4	432	670	1.27
1: 6	432	670	1.28
1: 8	432	669	1.28
1:10	432	665	1.29
1:50	432	664	1.29

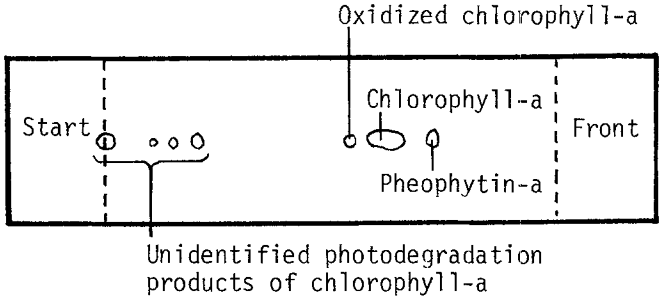

Fig. 4. Thin-layer chromatogram of chlorophyll *a* and its degradation products in the multilayers after irradiation of light developed in solvent system (*tert*-butyl alcohol/pentane/acetone=0.5:9:0.5, vol/vol/vol). The freshly prepared chlorophyll *a* multilayers before irradiation of light was dissolved in diethyl ether and the solution showed a single spot of chlorophyll *a* on a thin-layer chromatography developed by the same solvent system. 2.5×10 cm of commercial silical gel sheets (silica gel "Spotfilm" without fluorescence indicator, Tokyo Kasei, Japan) were used. Solutions of test materials in diethyl ether were spotted with a 2-µl micropipette 1.5 cm from the lower edge. High-performance liquid chromatographic test supported the thin-layer chromatographic observation

mains unsettled. The position of the spot for the oxidized Chl-*a* on the thin-layer chromatogram was behind the spot for Chl-*a*. Chl-*a* molecules in the pure Chl-*a* multilayers prepared at the surface pressures of 10.0, 15.0 and 20.0 mN m^{-1} were also chemically stable in air for at least 2 hr against irradiation. When the pure Chl-*a* multilayer prepared at the surface pressure of 16.5 mN m^{-1} was exposed in water vapor for 2 hr, the appearance of new band around 740 nm was observed. Leblanc and Chapados [13] have already found the same phenomena. They speculated that the appearance of the new band might be derived from the formation of the Chl-water complex. When the Chl-*a* multilayer thus treated was irradiated by light, Chl-*a* molecules in the multilayer were chemically more stable (for at least 90 min) than that in the untreated multilayer.

The multilayers consisting of the mixed monolayers containing Chl-*a* and PC at different molar ratios were also irradiated by the light in air. Chl-*a* molecules in the multilayers consisting of the mixed monolayers were chemically more stable (for at least 3 hr) against irradiation. Chl-*a* molecules in the multilayers prepared from the mixed monolayers containing Chl-*a* and PC at molar ratios of 1:1 and 1:2 were chemically most stable for at least 4 hr. Photodegradation products of Chl-*a* in these multilayers by the irradiation of the light for longer periods were confirmed as of the same species as in the case of the pure Chl-*a* multilayers, although the amount of the oxidized Chl-*a* formed in the mixed multilayers irradiated for the same time was relatively small compared to that formed in the irradiated pure Chl-*a* multilayer. When the irradiation was accomplished in nitrogen atmosphere, the oxidized Chl-*a* was not formed in the mixed multilayers. When the multilayer prepared at the surface pressure of the mother mixed monolayer (containing Chl-*a* and PC at molar ratio 1:2) of 16.5 mN m^{-1} was exposed in water vapor for 12 hr, the appearance of a new peak was observed around 700 nm as is shown in Fig. 5. Chl-*a* molecules in the multilayer thus treated were also chemically stable in air for at least 4 hr against irradiation.

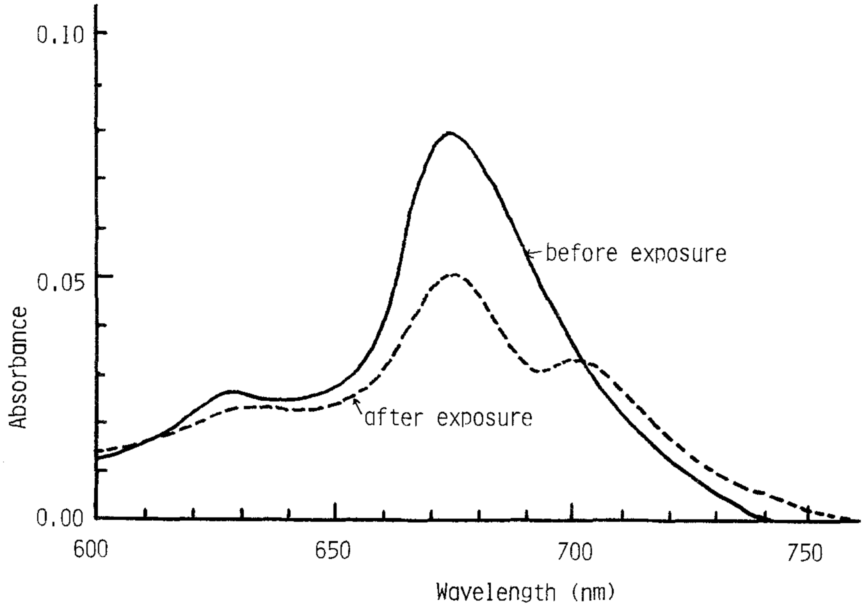

Fig. 5. Absorption spectra of twenty monolayers containing chlorophyll *a* and phosphatidylcholine before and after exposure in water vapor for 12 hr at 20 °C. The monolayers were spread from a 1:2 mixture and were deposited under a surface pressure of 16.5 mN m^{-1}

As described above, it has been confirmed that the lability of Chl-*a* molecules to photodegradation is dependent on the environment and the molecular arrangement or orientation. The lability is in the following order: the mixed mono- and multilayers < the pore Chl-*a* mono- and multilayers ≪ organic solvents.

It can be said that closely packed layers are playing an active role in protecting Chl-*a* from photodegradation and also that PC molecules in the layered structure are playing an active role from photobleaching as has been reported by Stillwell and Tien [17]. Phosphatidylcholine molecules are also incorporated in highly organized lamellar structure in vivo [14], in which photosynthetic primary photochemical reactions are performed. For this reason, it may be emphasized that PC molecules play an active role in protecting Chl-*a* molecules from photobleaching in vivo as well as in forming membrane structure.

Quantitative analysis of degradation products of Chl-*a* has not been performed in this study because some of the degradation products have not been identified and rates for the reaction from Chl-*a* to its degradation products have not been well controlled. Knowing the periods in which Chl-*a* molecules are chemically stable is very important in order to obtain meaningful results derived from Chl-*a*. In our previous report [8], it was showed that the appropriate application of chromatography is profitable for checking chemical stability and purity of Chl-*a* during the course of chemical and physical treatments. Experimental results presented in this report also may support the above statement.

We have not been able to elucidate any exact information on the molecular arrangement and orientation in multilayer structures. The finding that the absorption maxima shifted to longer wavelengths, when the mole fractions of Chl-*a* in the monolayers were increased, may be interpreted by the description that Chl-*a* and PC formed a nearly ideal two-dimensional solution. However, this interpretation cannot always be adopted in the case of high concentrations of Chl-*a* in the mixed monolayers. To elucidate the molecular arrangement and orientation in multilayers, physicochemical studies such as infra-red dichroism and fluorescence depolarization should be further required. We have found infra-red dichroism of Chl-*a* multilayer around free and asociated =CO absorptions [*to be published*]. However, we have not analyzed the dichroism quantitatively, as the =CO absorptions were strongly influenced by experimental conditions, which are difficult to control completely. Fluorescence derived from Chl-*a* molecules in mono- and multilayers were extremely weak to study fluorescence depolarization. These studies are now under way.

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