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FLUORESCENCE PROPERTIES OF CHLOROPHYLL *a* AND *b* MONOMOLECULAR FILMS AT THE AIR-WATER INTERFACE

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SUMMARY

The fluorescence properties of chlorophyll *a* and *b* monomolecular films at the air-water interface were measured by a high sensitivity fluorophotometer using the photon-counting method. The fluorescence intensity of chlorophyll molecules in monomolecular films in the absence of any diluents did not decrease simply with the mean distance of chlorophyll molecules. Over the range of the mean distances from 27 to 21 Å, three fluorescence components (peaks at 685, 695 and 715 nm) of chlorophyll *a* were observed. In the case of chlorophyll *b*, two fluorescence components (peaks at 667 and 685 nm) were observed over the range of the mean distances from 34 to 24 Å. When the mean distance was 18 Å, the short wavelength component of chlorophyll *b* disappeared, and only the long wavelength component was observed.

INTRODUCTION

It is generally accepted that the molecular state and the orientation of chlorophyll molecules are closely related to the mechanisms of the light absorption and the energy transfer in the primary process of photosynthesis. Purified chlorophylls *a* and *b* dissolved in organic solvents have only one absorption and fluorescence maximum at the red wavelength region. On the other hand, living chloroplasts have many absorption [1–3] and fluorescence [4–8] maxima derived from chlorophyll molecules. This fact has been postulated as evidence to indicate that these maxima might be due to different physical states of the chlorophyll molecules in chloroplasts, e.g. different and probably monolayer arrangements. The molecular structures of chlorophylls *a* and *b* are very similar to each other. Nevertheless, it is supposed that photophysical functions and molecular arrangements of chlorophylls *a* and *b* are very different in chloroplasts. Therefore, clarifying the differences in molecular level is important for elucidation of the primary process in photosynthesis. The chlorophyll molecules in

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monomolecular films at the air-water interface can be arranged in a variety of molecular states from the two-dimensional gas state to the solid state. Therefore, it is very important to measure the optical properties of the monomolecular films at the air-water interface at the various mean distances in order to elucidate the arrangements and the molecular states of chlorophyll molecules in vivo.

We have previously reported [9] that the very weak fluorescence emitted from the monomolecular films at the air-water interface can be measured directly and very accurately with a high sensitivity fluorophotometer using the photon-counting method. In this paper, the fluorescence properties of the chlorophyll *a* and chlorophyll *b* monomolecular films at the air-water interface at the various mean distances without any diluents are reported. The fluorescence maxima of chlorophyll molecules in the monomolecular films at the air-water interface and in vivo are discussed.

MATERIALS AND METHODS

Chlorophylls *a* and *b* used in these experiments were extracted from spinach leaves. Partially purified chlorophylls were obtained by the dioxane method as proposed by Iriyama et al. [10]. Then pure chlorophylls *a* and *b* were separated by chromatography on a sucrose column. The chlorophyll *a* used had absorption maxima at 428 and 661 nm, and the absorbance ratio of chlorophyll *a*, the red peak to the blue peak, was less than 1.3. The chlorophyll *b* had absorption maxima at 453 and 642 nm, and the absorbance ratio of chlorophyll *b*, the red peak to the blue peak, was less than 3.0. Before the experiments, the purity of chlorophyll *a* and *b* was checked spectroscopically in diethyl ether solution. All organic solvents purchased were reagent grade and further distilled before use. The water used was twice distilled in glass stills. The monobasic and dibasic potassium phosphate used in buffer solutions were purified with a Soxhlet extractor.

In a prevous paper [9], the apparatus for measuring very weak red fluorescence emitted from chlorophyll molecules in the monomolecular films at the air-water interface is reported in detail. Chlorophylls *a* and *b* dissolved in distilled benzene were spread on the air-water interface using a calibrated glass syringe. The Langmuir-type trough used was made of methacrylate and coated with paraffin. This trough was filled with a 10^{-3} M phosphate buffer solution of pH 8.0. The area illuminated at the air-water interface was about 0.5 cm^2. The excitation wavelength was 365 nm.

The emission spectra of the chlorophyll monomolecular films at the air-water interface were measured by changing the interference filters. The interference filters used were second order, with peak transmittance of about 40 % and width at half-maximum of 6–7 nm (Asahi Optics Co, Ltd.). All measurements are carried out at the room temperature of 23 ± 1 °C. The source light shutter was never kept open for more than the one minute necessary to make a reading. However, the fluorescence intensity was stable for 40 min within experimental errors under the irradiation of 5–10 μW exciting light. None of the fluorescence properties reported in this paper were dependent on the excitation light intensity. Fluorescence properties of two or three monomolecular films under the same conditions were measured with good reproducibility. The mean distance between nearest neighbor chlorophyll molecules was defined by the equation

$$R = \sqrt{A/\pi} \tag{1}$$

where A is the mean area occupied by a chlorophyll molecule, which is calculated from the content of chlorophyll molecules and the area spread on the air-water interface. The distance between chlorophyll molecules at the air-water interface was changed by changing the position of the paraffin-coated glass barriers.

RESULTS

The fluorescence intensity of chlorophyll *a* monomolecular films at the air-water interface against the mean distance of chlorophyll *a* molecules at the wavelength of 700 nm are illustrated in Fig. 1. The fluorescence intensity curves shown in Fig. 1 indicate that the chlorophyll *a* molecules in the monomolecular films do not behave according to simple concentration quenching. The fluorescence intensity shown was corrected for the molecular density of chlorophyll *a* per unit area. A fluorescence maximum was observed at the mean distance of 30 Å in the chlorophyll *a* monomolecular films. In Fig. 2, the fluorescence intensity of chlorophyll *b* monomolecular films against the mean distance of chlorophyll *b* molecules measured at the wavelength of 678 nm are shown. Like chlorophyll *a*, the chlorophyll *b* molecules do not behave according to simple concentration quenching.

The emission spectra of chlorophyll monomolecular films were then measured at various mean distances. The fluorescence intensity is corrected for the spectral

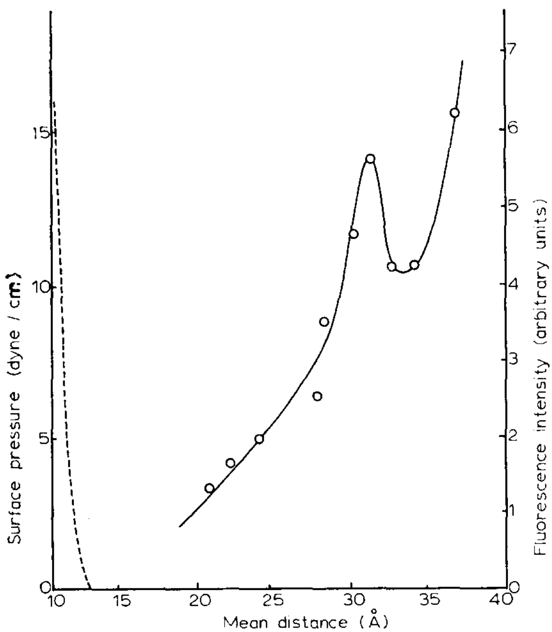

Fig. 1. Fluorescence intensity of chlorophyll *a* monomolecular films at the air-water interface against the mean distance. The force vs mean distance diagram of chlorophyll *a* is also indicated (- - -). The fluorescence intensity is corrected for chlorophyll *a* molecular density per unit area. pH 8.0 phosphate buffer.

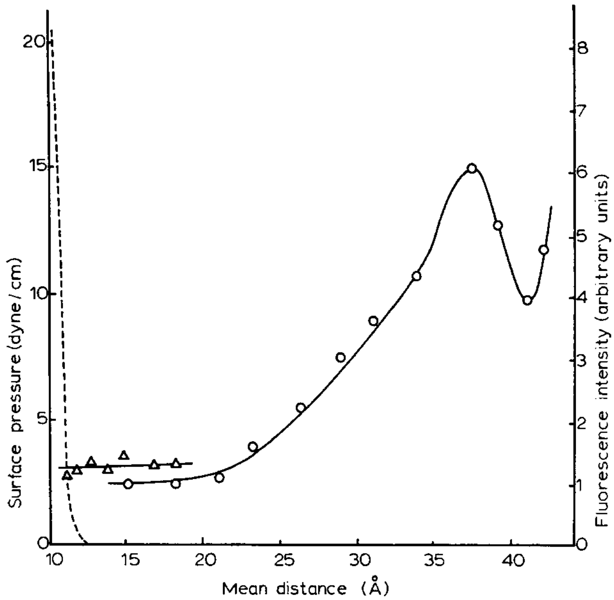

Fig. 2. Fluorescence intensity of chlorophyll *b* monomolecular films at the air water interface against the mean distance. The force vs mean distance diagram of chlorophyll *b* is drawn as a dashed line. The fluorescence intensity is corrected for chlorophyll *b* molecular density per unit area. pH 8.0 phosphate buffer. ○ and △, separate experiments.

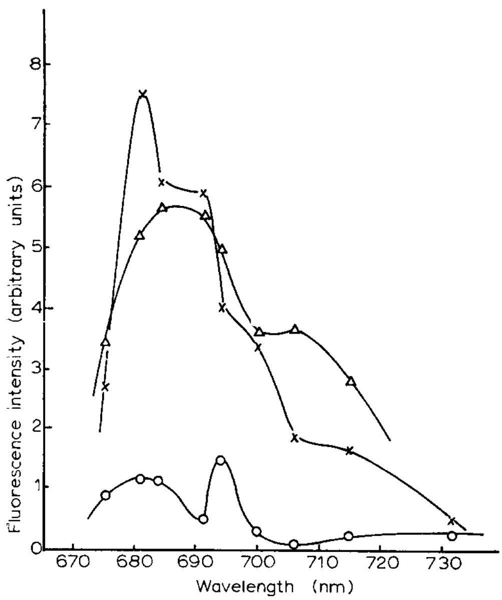

Fig. 3. Corrected emission spectra of chlorophyll *a* monomolecular films at various mean distances. ○, 27 Å; X, 23 Å; △, 21 Å. pH 8.0 phosphate buffer.

response of the photomultiplier, the optical properties of the filters used and the molecular density of chlorophyll per unit area.

The emission spectra of the chlorophyll *a* monomolecular films are shown in Fig. 3. When the mean distance was 27 Å, three fluorescence components with maxima at 685, 695 and 710–715 nm were observed. Fluorescence intensities of the components of 685 and 695 nm were nearly equal and the fluorescence intensity of the 710–715 nm component was weaker than that of other fluorescence components. When the mean distance was decreased to 23 Å, the total fluorescence intensity of the three components became stronger than that at 27 Å. The shorter the mean distance, the more the fluorescence intensity of the 710–715 nm component increased, and the more the intensities of other two components decreased. In the range of the mean distances from 27 to 21 Å, the fluorescence maxima of the chlorophyll *a* monomolecular films at the air-water interface were observed at 685, 695 and 710–715 nm. The intensity of each fluorescence maximum changed with the mean distance between chlorophyll *a* molecules at the air-water interface. The fluorescence intensity of the component having a peak of 710–715 nm increased with the mean distance. The total fluorescence intensity measured without filters on the wavelength range from 670 to 750 nm was decreased slightly (about 15 %) when the mean distance decreased from 27 to 21 Å.

Likewise, the emission spectra of the chlorophyll *b* monomolecular films measured at various mean distances are shown in Figs 4 and 5. When the mean distance was 34 Å, two fluorescence components with maxima at 667 and 685 nm

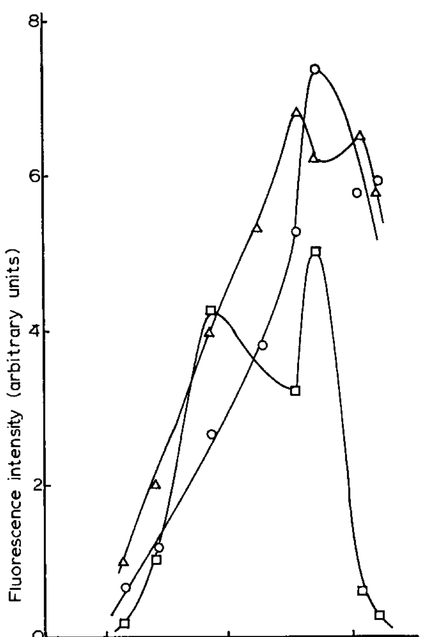

Fig. 4. Corrected emission spectra of chlorophyll *b* monomolecular films at various mean distances □, 34 Å; △, 24 Å; ○, 18 Å. pH 8.0 phosphate buffer.

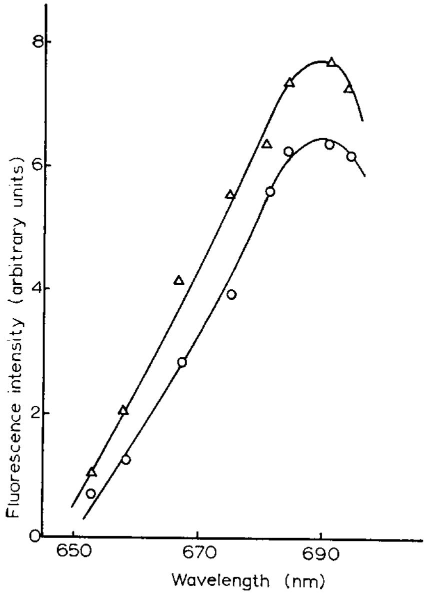

Fig. 5. Corrected emission spectra of chlorophyll *b* monomolecular films at the mean distances of 14 and 10 Å. △, 14 Å; ○, 10 Å. pH 8.0 phosphate buffer.

were observed. The fluorescence intensities of both components were nearly equal. The shorter the mean distance, the more the intensity of the long wavelength component increased, and the more the intensity of the short wavelength component decreased, as shown in Fig. 4. When the mean distance was 18 Å, the short wavelength component disappeared, and only the long-wavelength component was observed. When the mean distance was shorter than that of the solid state, the general features of the emission spectra of the chlorophyll *b* monomolecular films did not change and only the relative fluorescence intensity became weaker, as shown in Fig. 5. The total fluorescence intensity measured without filters in the wavelength range 650–700 nm decreased slightly (about 10 %) when the mean distance decreased from 34 to 10 Å.

Thus, the emission spectra of chlorophyll *a* and *b* monomolecular films at the air-water interface changed markedly with the mean distance between chlorophyll molecules.

DISCUSSION

Langmuir et al. [11] assumed that the fluorescence of the chlorophyll molecules was quenched when the spreading solvent was evaporated at the air-water interface. Tweet et al. [12] also adopted the assumption of concentration quenching. They measured the quenching curve of chlorophyll *a* molecules in the monomolecular films at the air-water interface with oleoyl alcohol as a two-dimensional diluent. They also reported [13] a shift of the emission spectra of mixed chlorophyll *a* films between the area fraction of chlorophyll *a*, 0.09 and 1.0. Almati et al. [14] measured the total fluorescence intensity of a chlorophyll *a* black film with lecithin against the mixing ratio. Their investigation showed that the total fluorescence maximum existed at the mixing ratio of about 0.3. However, until now, no one has succeeded in measuring in detail the fluorescence spectral changes of chlorophyll molecules in the monomolecular films at the air-water interface as a function of the mean distance of chlorophyll molecules.

Our previous paper [9] showed that the apparatus using the photon-counting method had a high sensitivity and a high accuracy. Therefore, we could measured directly the fluorescence spectra of the chlorophyll monomolecular films at the air-water interface at the various mean distances between chlorophyll molecules. The possible change of the molecular states of chlorophyll molecules as indicated by the fluorescence spectral changes cannot be detected by pressure-area measurements at a mean distance longer than about 12 Å because of very low surface pressure, as shown in Figs 1 and 2.

The fluorescence intensity of chlorophylls *a* and *b* molecules in the monomolecular films would be expected to become stronger and to have only one fluorescence maximum when the mean distance is extremely long. However, it is difficult to measure the fluorescence properties at a mean distance much greater than 40 Å for chlorophyll *a* and 34 Å for chlorophyll *b* because of the lability and the possible nonuniformity of the monomolecular films of chlorophylls *a* and *b* at the air-water interface.

The interference filters used were inadequate to determine the accurate positions of the fluorescence maxima, because the separation between the fluorescence maxima was narrow, and yet the width at half-maxima of the interference filters was rather wide (6–7 nm). Nevertheless, it is evident that the three fluorescence components (peaks at 685, 695 and 710–715 nm) of chlorophyll *a* and the two fluorescence components (peaks at 667 and 685 nm) of chlorophyll *b* exsisted in the monomolecular films at the air-water interface.

It has been reported that in chloroplasts chlorophyll *a* molecules have three fluorescence maxima [5, 8] and chlorophyll *b* molecules have one fluorescence maximum [4]. We observed the three fluorescence components of chlorophyll *a* molecules in monomolecular films at the air-water interface over the range of the mean distances from 27 to 21 Å. In the case of chlorophyll *b*, the two fluorescence components were observed in the monomolecular films at the air-water interface over the range of mean distances from 34 to 18 Å. The observed fluorescence maxima of chlorophyll *a* and *b* are summarized in Table I for comparison with those reported by other investigators [4–5, 8]. It seems that the three fluorescence maxima of chlorophyll *a* and the two fluorescence maxima of chlorophyll *b* in monomolecular films at the air-water interface correspond closely to those in chloroplasts. Moreover, the range of the distance between chlorophyll molecules over which these fluorescence maxima of chlorophylls *a* and *b* were observed correspond to those in chloroplasts, if a monolayer arrangement of chlorophyll molecules is assumed in the lamellar structure of chloroplasts. Molecular states similar to those of chlorophyll molecules in chloroplasts seem to be reproduced in the monomolecular films at the air-water interface. In the case chlorophyll *b*, only one fluorescence maximum was observed in chloroplasts, but in the monomolecular films at the air-water interface, two fluorescence maxima were observed. The longer wavelength maximum, having a peak of 685 nm in the monomolecular films, may be derived from a two-dimensional crystalline state of chlorophyll *b*. The different molecular states of chlorophyll molecules in vivo are represented

TABLE I

FLUORESCENCE MAXIMA OF CHLOROPHYLL *a* AND *b* MOLECULES IN VIVO AND IN THE MONOMOLECULAR FILMS AT THE AIR-WATER INTERFACE

Fluorescence maxima (nm)				
in vivo				in monomolecular films (these experiments)
	Brody and Brody [4]	Cho et al. [8]	Goedheer [5]	
Chlorophyll *a*	685	689	686	685
	695	698	696	695
	720	725	717	715
Chlorophyll *b*	660			667
				685

as different monomolecular arrangements of chlorophyll molecules having different mean distances.

We are confident that the monomolecular films at an air-water interface in the absence of any diluents are a very suitable system to investigate the arrangement of chlorophyll molecules. It is expected that the fluorescence measurements of chlorophyll *a* and *b* molecules using the photon-counting method provides a strong clue as to the molecular states of chlorophyll molecules, which should be closely related to the primary process of photosynthesis at the molecular level.

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