The Absorption Spectra of Monolayers of Fluorescent Probes and the Orientations of Their Chromophores

Kazunori MATSUKI* and Hideo FUKUTOME
Department of Physics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606
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The UV absorption spectra of monolayers of 12-(9-anthrylcarbonyloxy)octadecanoic acid (12-AS), 2-(9-anthrylcarbonyloxy)hexadecanoic acid (2-AP), and 2-octadecylamino-6-naphthalenesulfonic acid (ONS) were measured at normal incidence and the polarized spectra at 45° incidence. New peaks which were absent in the spectra of their solutions were observed in the polarized spectra at 45° incidence. The orientation of their chromophores in the monolayers were investigated by means of the dichroic ratios obtained from their polarized spectra. These monolayers were found to consist of H-aggregates where chromophores are oriented nearly perpendicularly to the monolayer plane and molecules in a monomer-like state where the chromophores are rather randomly oriented. The molecular axes of 12-AS, 2-AP, and ONS were found to be oriented uniaxially against the normals of their monolayer planes in the ranges 0—7°, 0—21°, and 0—34°, respectively.

Fluorescent probes have been widely used to study the behavior of lipids and proteins in natural and model membranes.[1] Cadenhead *et al.* showed that 12-(9-anthrylcarbonyloxy)octadecanoic acid (12-AS), 2-(9-anthrylcarbonyloxy)hexadecanoic acid (2-AP), and 2-octadecylamino-6-naphthalenesulfonic acid (ONS) form stable and condensed monolayers at the air-water interface.[2,3] Their chromophores will probably interact strongly in these monolayers. Therefore, their monolayer spectra are expected to differ remarkably from their solution spectra. The absorption spectra of these monolayers, however, have not been measured yet and information about the orientation of their chromophores in the monolayers is lacking although Teissie *et al.* studied the fluorescence of 12-AS monolayers.[4]

In this paper the polarized absorption spectra of monolayers of 12-AS, 2-AP, and ONS were measured in order to get information about the interactions and the orientations of their chromophores in monolayers.

Experimental

Materials. 12-AS, mp 79 °C (lit, 78.5 °C[2]) and crystalline dipalmitoyl-L-α-phosphatidylcholine (DPPC) were purchased from Sigma Chemicals Co., stearic acid (standard for elementary analysis) from Merck Co., and 2-AP, mp 96 °C (lit, 91—92 °C[2] and 94 °C[5]) from Molecular Probe Inc. ONS (Nakarai, S.P.-grade) was recrystallized from chloroform–methanol (2 : 1, v/v).[6] The purity of ONS was checked by TLC on precoated plates (Merck, Kieselgel 60 F_{254}) in chloroform–methanol (1 : 1, v/v). It revealed a single spot both under UV light to visualize fluorescent species and in iodine vapor to check nonfluorescent contaminants. All the above chemicals, except stearic acid, were stored in the dark about −20 °C in a desiccator. Hexane, ethanol, methanol, and chloroform were spectroscopic grade (Nakarai). Dichloroacetic acid (Nakarai, E.P.-grade) was distilled under vacuum. These chemicals were used without further purification unless otherwise stated. Twice-distilled water (pH=5.0—5.5) was used in all the experiments. Optically flat quartz plates (50×24×0.8 mm³) were obtained from Kōno Seisakusho (Kyoto).

Methods. The UV absorption spectra of 12-AS, 2-AP, and ONS in organic solvents were recorded on a UV-180 spectrophotometer (Shimadzu Co.). The absorption spectra of their monolayers were measured with a high-sensitivity spectrophotometer using the single-beam and sample in-sample out technique.[7] It was constructed in our laboratory.[8]

In the measurements of the polarized absorption spectra at 45° incidence, a polarizer (a Glan-Thomson prism made of calcite) was set before the plate in such a manner as to make the plane of polarization parallel to the plane of incidence (p-spectrum) or perpendicular to it (s-spectrum).[7]

The Blodgett method[9] was used for preparing spectroscopic samples of monolayers. A monolayer was spread on twice-distilled water at 23±1 °C by dropping a solution (5—10 mg/10 cm³). Spreading solvents were hexane–ethanol (10 : 1, molar ratio)[2] for 12-AS and 2-AP, and chloroform–dichloroacetic acid (9.6 : 0.4, v/v)[3] for ONS. Surface pressure was applied to the monolayer by using oleic acid (30 mN/m), ethyl myristate (20 mN/m), or a mixture of ethyl myristate and liquid paraffin (16 mN/m)[10] as a piston oil. One monolayer was transferred on each side of the lower half of the clean quartz plate.

The details of cleaning the quartz plate were described previously.[8]

Results and Discussion

In the high-sensitivity spectrophotometer with the single-beam and sample in-sample out technique used in this study, the measured absorption includes the apparent absorption due to the difference in reflectivity between the monolayer and the plate and to the inhomogeneousness in the quartz plate as described before.[8] Since 12-AS, 2-AP, and ONS have a stearic acid-like chain, the apparent absorption of their monolayers can be canceled out by subtracting the apparent absorption of a monolayer of stearic acid. In all the spectra shown in this paper this correction for the apparent absorption was made.

The polarized absorption spectra at normal and 45° incidences were measured in order to investigate the anisotropy of the monolayers. In the case of the normal incidence, the spectra of the monolayers were examined with the plane of polarization set at the angles 0°, ±45°, and 90° against the direction of the withdrawal of the plate from the monolayer at the air-water interface. All the monolayers examined showed no significant difference in the polarized spectra at normal incidence. This indicates that no anisotropy exists in the layer plane. In the case of the 45° incidence, however,

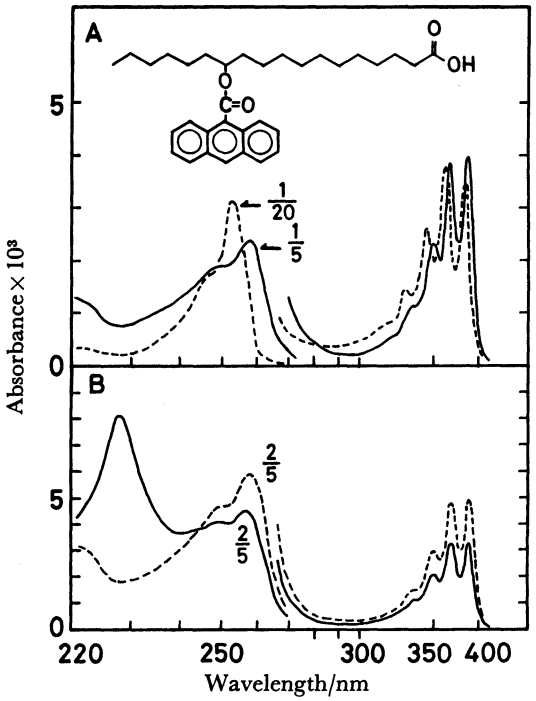

Fig. 1. Absorption spectra of the 12-AS monolayer spread on twice-distilled water and transferred to a quartz plate under the surface pressure of 16 mN/m. The correction for apparent absorptions was made as described in text.

(A) Absorption of the monolayer at normal incidence (n-spectrum) (——) and absorption in solution in an arbitrarily chosen scale (------). (B) Polarized spectra of the monolayer at 45° incidence. The plane of polarization was parallel to the plane of incidence (p-spectrum) (——) or perpendicular to it (s-spectrum) (------).

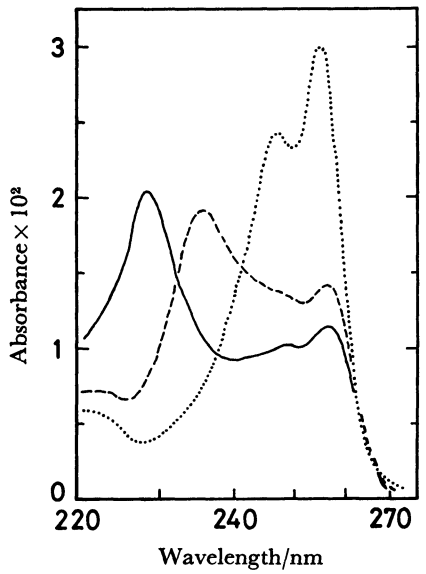

Fig. 2. P-Spectra of the monolayers of 12-AS mixed with DPPC. The monolayers were spread on twice-distilled water and transferred to a quartz plate under the surface pressure of 16 mN/m. The molar ratio of 12-AS to DPPC was 1 : 0 (——), 3 : 2 (------), or 1 : 4 (·····). The absorbances were normalized so as to give the same concentration of 12-AS per area.

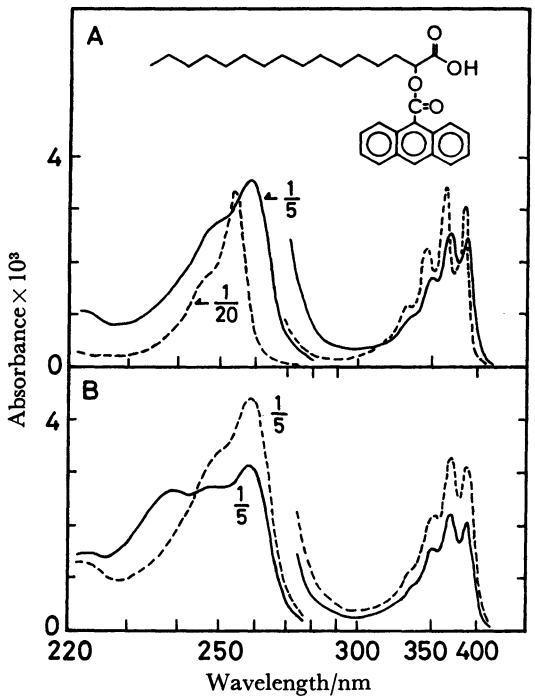

Fig. 3. Absorption spectra of the 2-AP monolayer spread on twice-distilled water and transferred to a quartz plate under the surface pressure of 20 mN/m. The notations are the same as those in Fig. 1.

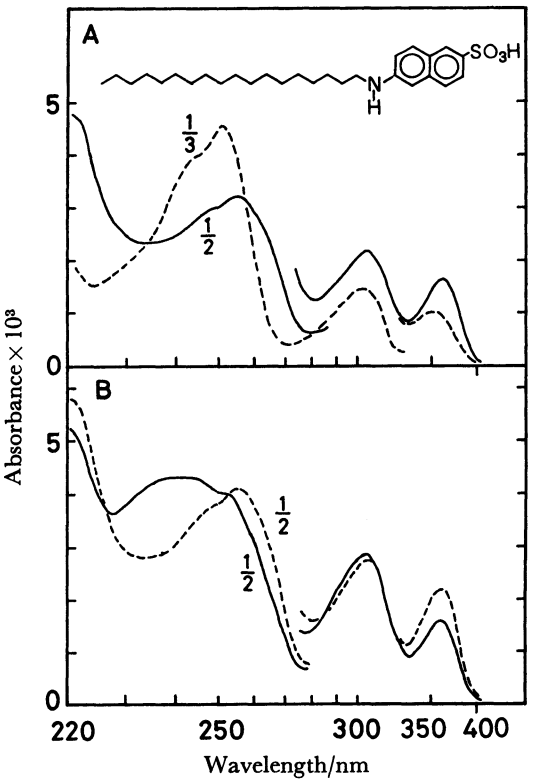

Fig. 4. Absorption spectra of the ONS monolayer spread on twice-distilled water and transferred to a quartz plate under the surface pressure of 30 mN/m. The notations are the same as those in Fig. 1.

remarkable differences between the p- and s-spectra were observed. These indicate that the molecules in all the monolayers are oriented uniaxially. The apparent

TABLE 1. SPECTRAL DATA AND ORIENTATION ANGLES OF THE MONOLAYERS

Compound	λ_{max}/nm		R_{app}[a]	R[a]	θ/°	γ/°
	Monolayer	Solution[b]				
12-AS	229	—	≈0	≈0	≈0	≈0
	257	253	1.306±0.002	1.094±0.002	60—62	
	333	328	—	—	—	
	348	343	1.447±0.008	1.215±0.007	70—73	0—7
	366	361	1.521±0.005	1.278±0.004	79—83	
	386	380	1.542±0.001	1.295±0.001	85—90	
2-AP	239	—	≈0	≈0	≈0	≈0
	258	254	1.418±0.008	1.187±0.007	68—75	
	334	329	—	—	—	
	350	344	1.435±0.003	1.205±0.002	70—76	0—21
	367	362	1.470±0.007	1.234±0.006	72—84	
	387	381	1.495±0.009	1.256±0.008	75—90	
ONS	241	—	≈0	≈0	≈0	≈0
	255	252	—	—	—	
	306	305	0.957±0.013	0.803±0.011	34—45	0—34
	360	351	1.400±0.010	1.176±0.009	66—90	

a) The average of the results for two samples prepared independently. b) Solvent: hexane for 12-AS and 2-AP, methanol for ONS.

dichroic ratio, $R_{app}=A_s/A_p$, was obtained from A_s and A_p, the absorbances of the s- and p-spectra, which were corrected for the apparent absorption as has been described above. The true dichroic ratio, R, corrected for the interference effect[7] was calculated from R_{app} as described before.[8]

Figures 1 to 4 show the observed absorption spectra in solution and those of the monolayers of 12-AS, 2-AP, and ONS which are corrected for the apparent absorption. Table 1 lists the positions of the absorption peaks in the solution and monolayer spectra and the apparent and true dichroic ratios. A new band which was absent in the solution spectra and in the spectra at the normal incidence appeared in all the p-spectra at the 45° incidence. This band seems to arise from the excitonic interaction among chromophores on formation of aggregates in the monolayers as will be discussed in detail later.

The absorption spectra of 12-AS and 2-AP in hexane closely resemble that of anthracene. The longer wavelength bands with fine vibronic structures can be assigned to the ^1L_a transition and the intense bands at shorter wavelength to the ^1B_b transition.[11]

The nonpolarized spectrum at the normal incidence (n-spectrum) of the 12-AS monolayer was similar to its solution spectrum though it was slightly red-shifted (Fig. 1). This shift appears to be due to a solvatochromic effect. In the polarized spectra at the 45° incidence, a new absorption band which was absent in the solution spectra appeared at 229 nm in the p-spectrum. The s-spectrum had no peak corresponding to this new band and showed the same pattern as the n-spectrum. This shows that the transition moment of the new 229 nm band is oriented nearly perpendicularly to the layer plane. The p-spectra of the monolayers of 12-AS mixed with DPPC were measured in order to clarify the origin of the 229 nm band. As can be seen from Fig. 2, the 229 nm band was shifted toward the 257 nm band and its absorption intensity was decreased whereas the intensity of the 257 nm band was increased as 12-AS was diluted with DPPC. The band at 333—386 nm showed no significant changes, and so these spectra are not shown in Fig. 2. The marked concentration dependence of the 229 nm band in the mixed monolayer of 12-AS and DPPC shows that it is a band blue-shifted from the ^1B_b band by the excitonic interaction on formation of aggregates where the chromophores are arranged regularly. The shift of the 229 nm band seen in Fig. 2 can be explained if the size of the aggregates is decreased as 12-AS in the monolayer is diluted.[12] The direction of the transition moment of the ^1B_b band of anthracene is parallel to its long axis according to the data obtained by Inoue *et al.*[13] The p-polarization of the 229 nm band shows that the aggregates formed in the monolayer are H-aggregates.[14] In those H-aggregates, the long axes of the anthracene moieties of 12-AS are oriented parallel to each other and perpendicularly to the layer plane. This explains the blue shift of the ^1B_b band in the H-aggregates.[12]

The 257 nm band in the 12-AS monolayer has the peak position and the band shape similar to the 253 nm band in solution, so it can be obviously assigned to the ^1B_b transition. Its polarization is small as seen from the true dichroic ratio given in Table 1. Its intensity increased as the intensity of the blue-shifted band in the H-aggregates decreased upon dilution of 12-AS. These observations show that the 257 nm band corresponds to the ^1B_b transition in 12-AS molecules with a different state of the chromophores from the H-aggregates. It is most likely that the chromophores in the state are oriented rather randomly. We call this state the monomer-like state. Therefore, the 12-AS monolayer appears to consist of the H-aggregates and the molecules in the monomer-like state. Heesemann reported absorption spectra similar to those in our study in the monolayers of surface-active azo and stilben dyes.[15] The H-aggregates in the 12-AS monolayer seem to correspond to the "microdomains" termed by

Teissie *et al.*[4]

The band at 333—386 nm in the 12-AS monolayer can be assigned to the ^1L_a transition because its peak positions and vibronic structure resemble those of 12-AS in solution. The ^1L_a band of the H-aggregates seems to be almost unchanged and superposed on that of the monomer-like state because no new peaks appeared near this wavelength region. The direction of the transition moment of the ^1L_a band is parallel to the short axis of anthracene and is orthogonal to the transition moment of the ^1B_b band according to Inoue *et al.*[13] This means that the transition moment of the 333—386 nm band is nearly parallel to the monolayer plane in the H-aggregates. The absence of observable excitonic shift in this band might mean that the short axes of the anthracene moieties are not regularly arranged but randomly oriented in the H-aggregates. However, we can not eliminate the possibility of a regular arrangement of the chromophores. The excitonic spectral shift is proportional to the square of the magnitude of the transition moment in the point dipole approximation of excitonic interaction.[16] The spectral shift of the ^1B_b band in the H-aggregates is 28 nm (=257 nm−229 nm). The magnitude of the transition moment of the ^1L_a band is found to be about 1/17 of that of the ^1B_b band from their absorbances in the solution spectrum. The spectral shift of the ^1L_a band in the H-aggregates is estimated from this relative magnitude of the transition moment to be less than 0.3 nm even if the transition moments are completely parallel arranged. Such a small shift can not be detected with our spectrophotometer even if there is no solvatochromic effect. Therefore, we can not discriminate which of the two possibilities is the case that the anthracene moieties in the H-aggregates are stacked or that their short axes are randomly oriented.

The chromophores in the 12-AS monolayer are oriented uniaxially as stated above. The orientation angles of the molecules were calculated by assuming the same model as used in a previous paper.[8] Let γ be the angle between the normal of the layer plane and the principal axis of the molecule and θ be the angle between the transition moment and the principal axis of the molecule. Then, the θ and γ angles to give a fixed value of the true dichroic ratio R make a curve in the θ-γ plane.[8] We show in Fig. 5 the θ-γ curves to give the observed values of R for the absorption peaks in the 12-AS monolayer. In the band at 229 nm, R can be regarded as ≈ 0 because there is no peak corresponding to it in the s-spectrum. The θ and γ angles clearly become $\approx 0°$ in this case, and so its curve is not shown in Fig. 5. The calculation for the peak at 333 nm was not done because it seems unreliable owing to the smallness of the absorbance and the presence of an overlap of the neighboring absorption bands. The dichroic ratio of the 257 nm band belongs only to the monomer-like state. In the 348-, 366-, and 386-nm peaks, it should be noted that their dichroic ratios are the averages of those of the H-aggregates and of the monomer-like state. It is difficult to separate the spectrum of the monomer-like state from that of the H-aggregates because of the overlap of their spectra. The γ angle

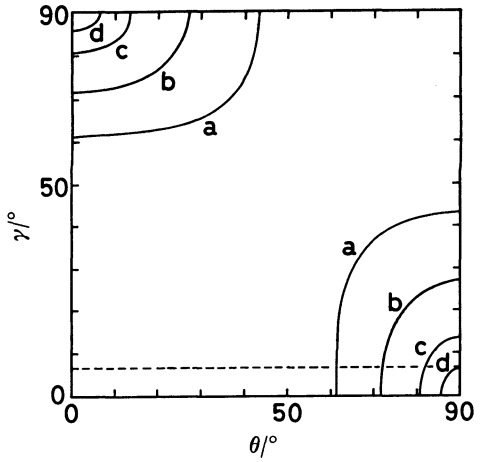

Fig. 5. The θ-γ curves to give the observed values of the true dichroic ratio R in the 12-AS monolayer. Curve a: for the 257 nm band, b: 348 nm band, c: 366 nm band, d: 386 nm band. The broken line indicates the upper limit of γ.

of the monomer-like state was then assumed to be equal to that of the H-aggregates. This means that the 12-AS monolayer is regarded as a homogeneous monolayer as long as fatty acid chains concern though it is a heterogeneous monolayer in chromophore arrangement. Then, the γ angle must have the same value for all the band. Hence, the θ's for each band are obtained from the points where each curve intersects the line of a constant value of γ in Fig. 5. The admissible ranges of γ are determined as 0—7° and 85—90° from the curve d where the permissible range of γ is the narrowest in the four curves. The range, γ=85—90°, is inadequate because it means that the alkyl chains of 12-AS molecules are almost parallel to the water surface. Therefore, we adopt the range, γ=0—7°. This is consistent with the result, $\gamma \approx 0°$, obtained from the polarization of the 229 nm band in the H-aggregates.

The admissible ranges of θ are summarized in Table 1. The ranges of θ for the vibronic peaks in the 348—386 nm band indicate that the direction of the transition moment of this band is roughly parallel to the short axis of the anthracene moiety in agreement with the ^1L_a assignment. Let us assume that the short axes of the anthracene moieties in the monomer-like state are isotropically distributed (R=1) and those in the H-aggregates are fully parallel to the layer plane. Then, the molar ratio of 12-AS molecules in the H-aggregates is estimated to be 98% from the observed R value of 1.295 for the 386 nm band. The dichroic ratio, R, of this band in the H-aggregates is assumed to be 1.299, the theoretical value for γ=0° and θ=90°. This high value of the molar ratio of the H-aggregates is unreasonable because the 257 nm band of the monomer-like state has a considerable absorption intensity. Therefore, the assumption of isotropic distribution of the short axes of the anthracene moieties in the monomer-like state is not valid and they are likely to be oriented much more parallel to the layer plane.

The admissible range of θ is 60—62° for the ^1B_b band at 257 nm. Cadenhead *et al.* suggested that the an-

thracene moiety of 12-AS is parallel to its alkyl chain at the area/molecule about 40 Å^2.[2] In the H-aggregates, the orientation of the anthracene moiety obtained from the polarized spectra agrees with their suggestion because $\theta \approx 0°$ for the 229 nm band. The θ angle obtained from the 257 nm band, however, indicates that the anthracene moiety fairly twists against the molecular axis in the monomer-like state. This suggests that the anthracene moiety makes an internal rotation around the –CO–C– bond when the molecules in the monomer-like state are transformed into the H-aggregates.

The n-spectrum of the 2-AP monolayer also showed a red-shift compared with its solution spectrum (Fig. 3). This shift may be ascribed to the solvatochromic effect. A new absorption band which was absent in the solution spectrum appeared at 239 nm in the p-spectrum at the 45° incidence but s-spectrum showed no peak corresponding to this band. This indicates that the transition moment of the new band is oriented nearly perpendicularly to the layer plane. The 239 nm band can be assigned to the excitonic band blue-shifted from the ^1B_b band at 258 nm similarly to the 229 nm band of 12-AS. The 2-AP monolayer also appears to consist of the H-aggregates and the molecules in the monomer-like state. The spectral shift of the chromophores in the H-aggregates is smaller than that in the 12-AS monolayer. This may be due to that the excitonic interaction among the chromophores of 2-AP is weaker than that in the 12-AS monolayer because 2-AP makes a more expanded monolayer than 12-AS.[2] The 334—387 nm band may be assigned to the ^1L_a transition. The ^1L_a band of the H-aggregates appears to be almost unchanged and superposed on that of the monomer-like state because no new peaks appeared near this wavelength region. This is the situation also similar to the 12-AS case.

The orientation angles of the molecules in the 2-AP monolayer were calculated by the similar manner to the 12-AS case. In the 239 nm band, R can be regarded as ≈ 0 because there is no peak corresponding to it in the s-spectrum. Hence, γ and θ in this band should be $\approx 0°$. The calculation for the peak at 334 nm was not done for the same reason as the peak at 333 nm in the 12-AS molayer. For the bands at 258-, 350-, 367-, and 387-nm, the admissible ranges or γ and θ were estimated by drawing the θ-γ curves which give the observed values of R. These curves are very similar to those in Fig. 5, and they are not shown. The γ angle of the monomer-like state was also assumed to be equal to that of the H-aggregates. Then, we obtain the admissible ranges of γ as 0—21° and 75—90°. We adopt the former range as the physically reasonable one. The admissible ranges of θ are summarized in Table 1. The ranges of θ for the peaks in the 350—387 nm band indicate that the direction of their transition moments are roughly parallel to the short axis of the anthracene moiety. It should also be noted that the dichroic ratio of a peak in the band is an average of those of the H-aggregates and of the monomer-like state. The assumption of completely random distribution of the transition moments in the monomer-like state again leads to an unreasonably high fraction of the H-aggregates, 86%, from the observed R value of the 387 nm band, 1.256. Hence, the short axes of the anthracene moieties in the monomer-like state are not distributed isotropically but more parallel to the layer plane.

The range of θ is 68—75° for the 258 nm band but $\approx 0°$ for the 239 nm band. This suggests that the anthracene moiety of 2-AP also makes an internal rotation around the –CO–C– bond at the transformation from the monomer-like state to the H-aggregates. The anthracene moiety of 2-AP is very close to its hydrophilic group. The internal rotation of the anthracene moiety is likely to be sterically hindered by the water surface especially when $\theta \approx 0°$. Therefore, formation of H-aggregates seems to be harder in the 2-AP monolayer than in the 12-AS monolayer. The relative intensity of the 239 nm band to the 258 nm band in the 2-AP monolayer is smaller than that of the 229 nm band to the 257 nm band in the 12-AS monolayer (Figs. 1 and 3). This means that the fraction of the H-aggregates in the 2-AP monolayer is smaller than that in the 12-AS monolayer. This observation is consistent with the above mentioned structural feature of the 2-AP molecule.

The absorption spectra of the ONS monolayer are shown in Fig. 4. The solvent for the solution spectra was methanol because ONS was almost insoluble in hexane. A new absorption peak which was absent in the solution spectrum also appeared at 241 nm in the p-spectrum of the ONS monolayer. The transition moment of this band seems to be oriented nearly perpendicularly to the layer plane because its s-spectrum contained no peak corresponding to it. The peak at 241 nm was shifted toward the 255 nm band as the ONS monolayer was diluted with DPPC. Therefore, the 241 nm band is due to the H-aggregates and the ONS monolayer consists of the H-aggregates and the molecules in the monomer-like state similarly to the 12-AS and 2-AP monolayers. The 306- and 360- nm bands are little changed from those of the monomer-like state on formation of the H-aggregates because no new peak appeared near these bands.

The orientation angles of γ and θ in the ONS monolayer were estimated by the similar manner as above. In the 241 nm band, R can be regarded as ≈ 0 because there is no peak corresponding to it in the s-spectrum, and γ and θ in the H-aggregates are $\approx 0°$. The calculation for the 255 nm band was not done because its dichroic ratio could not be reliably measured owing to a large overlap with the 241 nm band in the p-spectrum. The orientation angles were tentatively estimated from the observed R values of the 306- and 360-nm bands. The γ angle in the monomer-like state and that in the H-aggregates were assumed to be the same. The θ-γ curves which give R's of the two bands are shown in Fig. 6. The admissible range of γ is estimated to be 0—34° from the curve b. The ranges of θ for the 306- and 360-nm bands are obtained as 34—45° and 66—90°, respectively. Badley *et al.* estimated θ from comparison of related compounds as 55° (=90°—35°) for the 360 nm band.[17] Their estimation is consistent with ours if the structure of ONS with respect to the bond C–N–C is as shown in Fig. 4. The curves in Fig. 6, however, were obtained from the

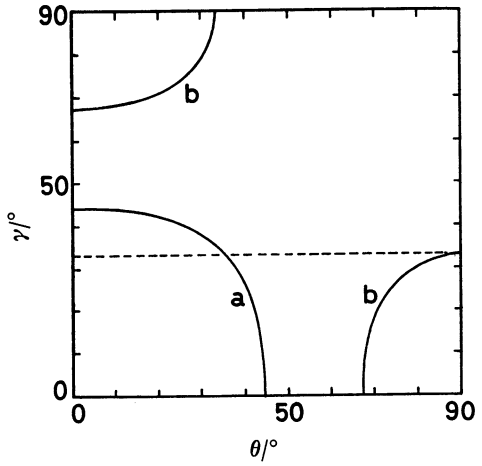

Fig. 6. The θ-γ curves to give the observed values of the true dichroic ratio R in the ONS monolayer. Curve a: for the 306 nm band, b: 360 nm band. The broken line indicates the upper limit of γ.

observed values of R which were the averages for the H-aggregates and the monomer-like state. Since no theoretical and experimental data about the directions of the transition moment of the chromophore of ONS are available, it is difficult to get further information about the orientation of the chromophores in the monomer-like state. By the way, the chromophore of ONS itself is the hydrophilic group at the end of the molecule. Another model for the orientation of ONS molecules is possible where their alkyl chains are randomly oriented independently of the uniaxial arrangement of their chromophores. The ONS monolayer, however, was transferred to the quartz plate under the surface pressure of 30 mN/m where the pressure-area isotherm of the ONS monolayer was steeply rising.[3] It seems unlikely that the alkyl chains in the ONS monolayer are randomly oriented because the surface pressure of the monolayer in such a steeply rising phase of the pressure-area isotherm is mostly produced by solid-like packing of long chains of surface-active molecules. Therefore, the model used by us seems to be plausible.

The pure monolayers of 12-AS, 2-AP, and ONS emitted blue fluorescence when excited by UV light. It was visible even to the naked eye. The fluorescence may provide further information about the H-aggregates and the monomer-like state in these monolayers. Roberts *et al.* reported that Langmuir films of short-chain derivatives of anthracene diplayed interesting electric conduction effects.[18] Electrical properties of 12-AS and 2-AP monolayers may also be worthy of a study.

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