Liposomal Membranes. IX. Fluorescence Depolarization Studies on *N*-Dansylhexadecylamine in Liposomal Bilayers

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A fluorescent probe, *N*-dansylhexadecylamine (DSHA), was found to aggregate in aqueous media by monitoring the emission maximum, relative intensity, and polarization of fluorescence as functions of solvent polarity and probe concentration. In aqueous methanol, the *critical solvent polarity* for DSHA at a given concentration to form self-aggregates corresponded to 57% (v/v) aqueous methanol, while the *critical aggregate concentration* for the probe was 2.3×10^{-6} M in 50% aqueous methanol. In liposomal bilayers, the dansyl moiety seemed to be localized in a polar region close to the surface of membranes. Fluorescence characteristics of DSHA in liposomes as a function of incubation temperature revealed the phase transition of liposomal bilayers, which was almost consistent with those previously estimated by other methods. As the salt concentration was increased up to about 1.5 M, DSHA in liposomes underwent the phase separation and formed self-aggregates even in liposomal bilayers.

In recent years fluorescence techniques have been extensively applied to study on the dynamic and static nature of biological membranes,[1] liposomes,[2] and aqueous[3] or reversed micelles.[4] Since the rotation rate of fluorescent probes closely relates to the resistance offered by the microenvironment against the mobility of the probes,[5] fluorescence depolarization provides useful information about the apparent "microviscosity" in terms of the environmental resistance. Shinitzky and Barenholz have recently studied on the fluidity and phase transition characteristics of single-walled and multilamellar liposomes of synthetic phospholipids using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe to know the nature of hydrophobic domain of liposomal bilayers.[6,7] On the other hand, to obtain the information about the surface of membranes 8-anilino-1-naphthalenesulfonate (ANS) has been employed.[8] Romero *et al.* adopted *N*-dansyloctadecylamine(DSOA) to obtain a knowledge about the less polar region of phospholipid bilayers,[9] since the fluorophore is localized in the vicinity of the phospholipid ester moiety.[10] The present studies have been undertaken using *N*-dansylhexadecylamine (DSHA) to measure the fluidity and microenvironment of the region close to the hydrophilic surface of bilayers as a function of incubation temperature or ionic strength. Besides these problems, in conjunction with Nagaraj and Balaram's postulate that the dansylated hydrophobic peptides aggregate in water,[11] we have studied also on the self-aggregation of DSHA by measuring fluorescence spectra and depolarization at very low concentrations in aqueous media and in bilayers as well.

Experimental

Materials. Egg yolk lecithin (egg L) was isolated and purified from fresh egg yolk as described before.[12,13] Dipalmitoyl-DL-α-phosphatidylcholine (DPPC) was purchased from Sigma Chemical Co., St. Louis, Mo. Phospholipids employed were found to be pure on TLC (a precoated silica gel plate, Spotfilm, Tokyo Kasei, Tokyo) as developed with chloroform/methanol/water (65 : 25 : 4, by vol).[12,13] *N*-Dansylhexadecylamine was prepared according to the method described in literature[9,14] with minor modification. A mixture of hexadecylamine (482 mg, 2.0 mmol) and dansyl chloride (593 mg, 2.1 mmol) was refluxed for 14 h in 20 ml of chloroform containing 3.0 ml of triethylamine. The resulting reaction mixture was washed six times with 50 ml of 10% aqueous citric acid and then twice with 50 ml of water, and the chloroform layer was dried over anhydrous magnesium sulfate. Removing off the solvent *in vacuo* gave a pale yellow crystalline mass, which was recrystallized from ether; yield, 540 mg (57%); mp 59—61 °C. Found: C, 70.16; H, 9.88; N, 5.77%. Calcd for $C_{28}H_{46}N_2O_2S$: C, 70.85; H, 9.77; N, 5.90%. IR (KBr): ν_{NH}, 3290; ν_{C-H}, 2930, 2860, 1592; ν_{S-O}, 1320 and 1150 cm^{-1}. Other organic and inorganic reagents were commercially available as analytical grade and used without further purification.

Preparation of Liposomes. Single-walled liposomes of egg L and DPPC were formed and isolated by the same method as that described previously.[12,15] Gel-filtration was carried out on a Sepharose 4B column (1.8×38 cm) equilibrated in water containing a given amount of sodium chloride. The concentration of liposomal suspension was determined as inorganic phosphate according to Allen's procedure.[16] The molar ratio of phospholipid to DSHA was in a range of 250—300.

Fluorescence and Depolarized Fluorescence Measurements. Fluorescence spectra and depolarization were measured by the essentially same procedures as those described before.[17,18] All the spectral measurements were run on a Hitachi 650-10S fluorospectrophotometer equipped with a thermoregulated cell compartment connecting to a Toyo Thermo Electric TE-104S.

The fluorescence polarization p is calculated by Eq. 1:

$$p = \frac{I_{VV} - C_f I_{VH}}{I_{VV} + C_f I_{VH}}, \quad (1)$$

where I is the fluorescence intensity and subscripts V and H refer to the vertical and horizontal orientations of the excitation (first) and analyzer (second) polarizers, respectively. C_f ($=I_{HV}/I_{HH}$) is the grating correction factor.[19] Depolarization measurements were run on a Union Giken fluorescence polarization spectrophotometer FS-501S using a sharp cut-filter Y-46 (Hoya Glass Works, Tokyo), of which cell compartment was connected to a Komatsu-Yamato Coolnics Model CTR-120. A Sord Microcomputer M 200 Mark II system was adopted to control the measurement conditions and to collect all the data.

Results and Discussion

Fluorescence Characteristics of DSHA in Homogeneous System. Both the emission maximum and intensity of DSHA were highly sensitive to solvents adopted as

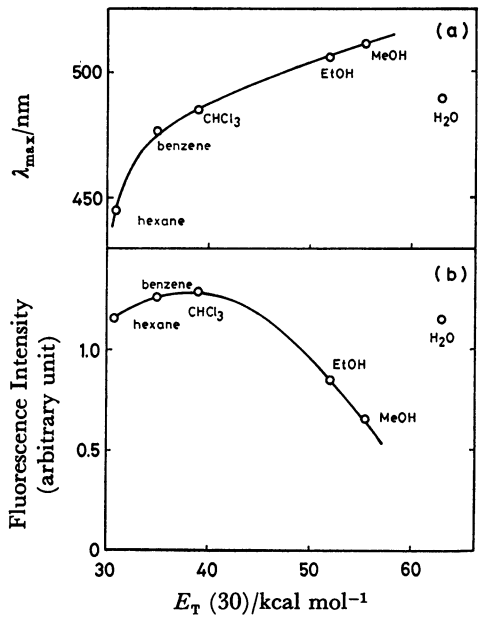

Fig. 1. Emission maximum (a) and relative intensity (b) of fluorescence from DSHA (8.6×10^{-6} M (1M=1 mol dm^{-3})) as a function of solvent polarity at 25.0 °C.

shown in Fig. 1. In apolar solvents, increasing the solvent polarity brings about a red shift of the maximum and a hypsochromic effect on the intensity. Interestingly, on the other hand, in polar protic solvents such as alcohols and water further increasing the solvent polarity causes a decrease in the intensity. In any event, the fluorescence characteristics of DSHA in water is rather different from those in organic solvents (Fig. 1).[9]

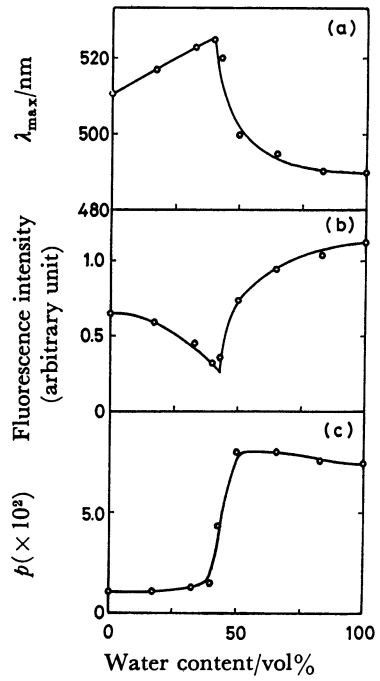

Fig. 2. Emission maximum (a), relative intensity (b), and polarization (c) of fluorescence from DSHA (8.6×10^{-6} M) in methanol as a function of water content at 25.0 °C.

When DSHA is excited at 340 nm in water, it emits the fluorescence at 487 nm. At first glance, this suggests that the microenvironment around the dansyl moiety of DSHA is close to that with the polarity of chloroform (Fig. 1). This is very interesting but unlikely. Figure 2 shows the maxima of fluorescence from DSHA in methanol as a function of water content. Up to 40% of water, the emission maximum first shifts toward a longer wavelength with an increase in the solvent polarity and further increasing the water content drastically shifts the maximum toward a shorter wavelength. Above 80% of water, it becomes almost constant (Fig. 2). The blue shift of the maximum means the translocation of the dansyl fluorophore from a polar environment to a less polar one, which means that DSHA may entangle by itself or form aggregates in aqueous media. On the basis of an evidence that a fluorophore-labeled peptide shows a blue shift of the emission maxima in an aqueous solution, Nagarj and Balaram have previously postulated an idea that peptides form micelles in water.[11] In order to confirm the self-aggregation of DSHA in aqueous media, hence, the fluorescence polarization p in methanol as a function of water content was measured (Fig. 3-c). The change of the p-value as a function of water content in methanol closely correlates with that of the emission maximum (Fig. 2-a). This suggests that DSHA molecules start to aggregate in methanol containing 43% (v/v) of water, giving rise to a decrease in the mobility. This point may be called the *critical solvent polarity* for the self-aggregation of DSHA at a given concentration. The relative intensity of fluorescence emission as a function of water content also reveals evidence for the aggregate formation of DSHA (Fig. 2-b). The self-aggregation is effected also by the DSHA concentration itself: for example, in 50% aqueous methanol the *critical aggregate concentration* for DSHA was found to be about 2.3×10^{-6} M by measuring the fluorescence spectra and depolarization. The *critical aggregate concentration* in methanol decreases with an increase in the water content.

Microenvironment around DSHA in Liposomal Bilayers. Various techniques such as NMR,[20,21] ESR,[22] and fluorescence spectroscopies[1,23] have been utilized so far to obtain information about the dynamic nature of lipid membranes. On the basis of the above results in homogeneous system where DSHA is sensitive enough to know the microscopic polarity and viscosity around the probe, in this work the fluorescence characteristics of DSHA in the liposomal bilayers were investigated. The emission maximum of DSHA in single-walled liposomes of egg L was 518 nm, while that in DPPC liposomes was 512 nm at 25.0 °C as seen in Fig. 3. The slight difference between the two liposomes must come from the difference in the state of bilayers at the temperature adopted; that is, single-walled liposomes of egg L are in a liquid-crystalline state,[24] while DPPC liposomes are in a gel state at 25.0 °C. In any event, using the ruler established for the microenvironmental polarity (Fig. 1), for both liposomes the fluorescent moiety of DSHA seems to be located in an environment with a polarity close to that of methanol. This means that the

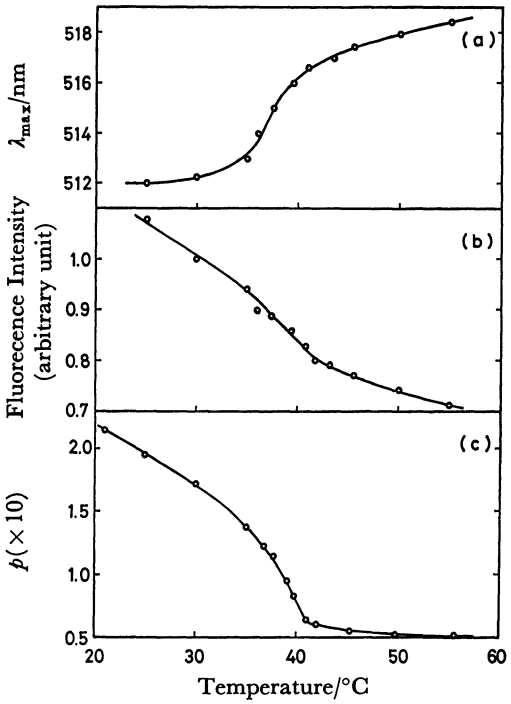

Fig. 3. Emission maximum (a), relative intensity (b), and polarization (c) of fluorescence from DSHA (4.0×10^{-6} M) as a function of incubation temperature in DPPC single-walled liposomes (8.0×10^{-4} M) dispersed in 0.1 M aqueous sodium chloride solution.

dansyl moiety is in a region close to the surface of liposomal bilayers.[9] At 55.0 °C the emission maximum of DSHA in DPPC single-walled liposomes shifts to 518.5 nm. At the temperature, DPPC liposomes are in a liquid crystalline state.[25]

Figures 3-a and -b show the maximum and relative intensity of fluorescence from DSHA in DPPC single-walled liposomes as a function of incubation temperature. Raising the incubation temperature of liposome suspension causes a red shift of the emission maximum and a simultaneous decrease in the quantum yield. Especially at temperatures between 35 and 45 °C, an abrupt change in the fluorescence characteristics was observed. The temperature-emission maximum profile reveals the phase transition between the gel and liquid crystalline states of bilayers (Fig. 3-a). Table 1 gives the phase transition temperatures determined by different techniques for DPPC liposomes, which indicates

Table 1. Phase transition temperature of DPPC single-walled liposome estimated by various techniques

Method	Phase transition temp/°C	Reference
(1) DSHA fluorescence polarization	38.4±0.5	this work
(2) DSHA fluorescence spectrum	39.0±0.5	this work
(3) Dilatometry	39	26
(4) DPH fluorescence polarization	36.4±0.5	7
(5) DPDL excimer formation	36.7±0.5	17
(6) Differential scanning calorimetry	36.9±0.9	25

that the phase transition temperature estimated in this work is in good agreement with those by other methods. As expected,[32] although the fluorescent moiety of the probe is located in the vicinity of the membrane surface, it is considerably sensitive to the phase transition of the hydrophobic domain. Table 1 also reveals that there may exist a temperature lag in the phase transition between hydrophilic and hydrophobic parts of bilayers: Methods 1—3 may reflect mostly the phase transition at the surface of bilayers, while Methods 4—6 at the deep hydrophobic domain. The most acceptable model for the conformation of phospholipid headgroups is that the headgroups lie parallel to the surface of bilayers and engage to the intermolecular electrostatic interaction with neighboring phospholipids.[27] It is well known that the motion and conformation of lecithin headgroup also change with the phase transition.[28,29] Above the phase transition temperature, the inter-headgroup interaction of lecithins is weakened and the distance between phosphorous atom and choline methyl group is far apart,[30,31] resulting in an increase in the hydration of the membrane surface.[27,32] Therefore, the red shift of emission maximum of DSHA is interpreted in terms of the increase in the polarity of the microenvironment around the probe. The correlation between the incubation temperature and the relative intensity of fluorescence was consistent with that between the temperature and the emission maximum (Fig. 3-b).

In order to monitor the mobility of the probe in egg L and DPPC single-walled liposomes, the fluorescence polarization p was measured (Figs. 3-c and 4). The increase in the fluidity of lipid membrane with raising the incubation temperature is reflected on the increase in the mobility of DSHA in bilayers. The fluorescence polarization-temperature profile also revealed the exsistence of a phase transition in the DPPC liposomes (Table 1 and Fig. 3-c). For the egg L liposomes, on the other hand, no abrupt change in the profile is observed (Fig. 4), since the egg L liposomes are in a liquid crystalline state over the temperature range adopted.[24]

The effect of ionic strength in the bulk aqueous phase on the fluorescence characteristics of DSHA in liposomal bilayers is of much interest. Above and below 1.5 M

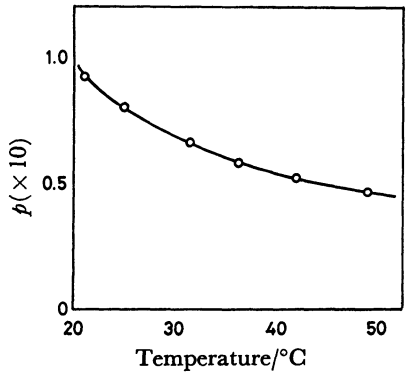

Fig. 4. Fluorescence polarization of DSHA (3.5×10^{-6} M) in egg L single-walled liposomes (8.0×10^{-4} M) dispersed in 0.1 M aqueous sodium chloride solution as a function of incubation temperature.

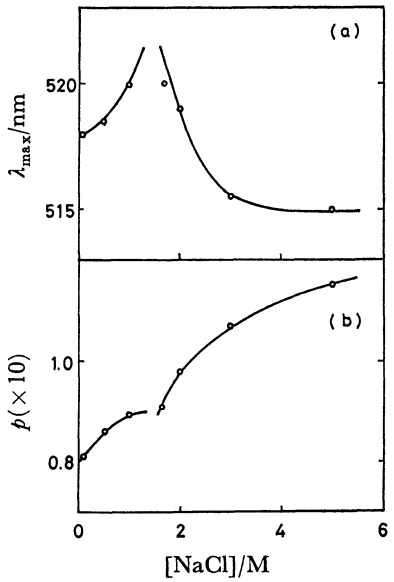

Fig. 5. Effect of the salt concentration in bulk aqueous phase on the emission maximum (a) and polarization (b) of fluorescence from DSHA intercalated in egg L liposomes (8.0×10^{-4} M) at 25.0 °C.

of the salt concentration the fluorescence characteristics of DSHA in liposomes are completely different from each other (Fig. 5). The point of 1.5 M salt concentration seems to be a boundary in the change of state for the surface of bilayers. The emission maximum shifts sharply toward a longer wavelength with increasing the ionic strength up to 1.5 M. A further increase causes oppositely a drastic blue shift of the emission maximum. On the other hand, increasing the ionic strength gradually increases the fluorescence polarization. However, the fashion of change in the polarization differs below and above the boundary of the ionic strength.

Träuble and Eibl have previously shown that an increase in the ionic strength with monovalent cations tends to fluidize the bilayer structure.[33] And they have postulated that the phase transition from the ordered state to the fluid one in charged bilayers is accompanied by a decrease in electrostatic free energy mainly as a result of bilayer expansion.[33] On the other hand, deuterium NMR studies of water bound to lecithin have indicated that hydration of the surface of bilayers increases with increasing the ionic strength in the bulk aqueous phase.[27,34] As the salt concentration is increased, the inter-headgroup interactions may be weakened, either by binding of the salt to the polar headgroups or by the increase in ionic strength, which results in the binding of more water molecules to the surface.[27] If this is the case, the microscopic polarity in the vicinity of the headgroup of lecithins will increase as the salt concentration is increased. This is consistent with the change in the emission maximum with increasing ionic strength up to 1.5 M. More hydration should bring about a blue shift of the emission maximum. Thus, the emission maximum change as a function of ionic strength closely resembles that as a function of water content in methanol (Fig. 2-a and Fig. 5-a). On the other hand, the increase in the polarity around the probe by penetration of more water molecules will cause a phase separation and self-aggregation of the probe just like that in bulk aqueous media. This will lead to a decrease in the mobility of the probe even in bilayers and hence an increase in the fluorescence polarization. The present findings reveal that neutral and less polar substances intercalated in liposomal bilayers must undergo the phase separation in bilayers when the salt concentration in bulk aqueous phase is largely increased.

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