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Liposomal Membranes. 2. Synthesis of a Novel Pyrene-Labeled Lecithin and Structural Studies on Liposomal Bilayers

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Abstract: A novel pyrene-labeled phosphatidylcholine, 1,2-bis[ω-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphorylcholine (DPDL), was synthesized for use in structural studies on the liposomal bilayers. From the fluorescence measurements on DPDL incorporated into the phospholipid bilayers as well as in organic solvents and aqueous buffered solutions, the following interesting information was obtained. (1) The probe DPDL is intercalated in a well-ordered fashion in the very hydrophobic domain of egg and dipalmitoylphosphatidylcholine bilayers. (2) Without cholesterol and/or below the phase-transition temperature, DPDL is arranged with the lipid-separated conformation in the shrunk liposomal bilayers (Figure 9d). (3) Increasing the mobility of lipid molecules by the addition of cholesterol and/or by elevating temperatures causes a conformational change of DPDL in the bilayers from the lipid-separated conformation to the intimate one (Figure 9c).

In connection with the role of biological membranes as the barrier against permeation of materials or the suitable domain for the membrane-bound enzymes, an understanding of the static and dynamic structures of lipid model membranes is important. To date a considerable amount of data has been accumulated about the lipid bilayer structure by the use of various techniques such as NMR,[1-3] DSC,[4] ESR,[5,6] or X-ray diffraction.[4,7] In order to obtain more detailed information about the structure and microenvironment of the bilayers, it is necessary to utilize such a probe that has more resemblance in the structure to the natural phospholipids and can sit in good order in the lipid bilayers. For this reason the probe which is covalently bound to the suitable positions of the lecithin molecule seems more preferable, as has been shown in the studies with the spin-labeled phospholipids[1,8] or the fluorescent phosphatidylcholine.[9]

Fluorescent probe techniques have been extensively applied to problems involving the fluidity and phase transition,[9,10] mobility and lateral diffusion,[11,12] polarity,[13,14] or surface potential of phospholipid bilayers.[15] However, the previously used fluorescent probes such as 8-anilino-1-naphthalenesulfonate (ANS),[16,17] anthroyl fatty acid derivatives,[18] pyrenes,[14,19] 1,6-diphenyl-1,3,5-hexatriene (DPH),[9,11] or polyene fatty acids[10] had serious drawbacks together with their advantages. For example, some of these probes such as pyrene or DPH, which have less resemblance to the natural phospholipids, may disturb the intact structure of lipid bilayers. In addition, since they are not linked covalently to the lecithin they have considerable freedom to move in the bilayers. Hence, they may easily change their location and orientation when the conditions such as composition of the membrane and temperature are varied.[9,11] Waggoner and Stryer employed a covalently fluorescent probe-labeled lipid, dansylphosphatidylethanolamine, but it only gave information about the region close to the membrane surface since the dansyl group was bound on the polar head group of the lecithin.[13] A possible reason that the use of covalently probe-labeled lecithins has been rather limited is the relative difficulty of the chemical synthesis of such lipids, especially when the probe is introduced to the fatty acid chains.[10,20]

We have designed a novel, covalently pyrene-labeled lecithin, 1,2-bis[ω-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphorylcholine (DPDL), to get information about the hydrophobic interior of liposomal bilayers. In order for the probe to fit best in the dipalmitoylphosphatidylcholine bilayers, the length of the polymethylene chains of the fatty acid residues is critical, and the appropriate length was chosen with the aid of a Corey-Pauling-Koltun (CPK) model (Figure 1).

Levine et al. have demonstrated by ^{13}C NMR spin-lattice relaxation measurements with covalently spin-labeled lipids that the lipid molecules are most tightly packed at the glycerol group.[1] The same conclusion has also been reached by studies on the lateral diffusion of the phospholipid molecules in bilayers.[21] Judging from these previous results, DPDL will be intercalated in a well-ordered fashion within the phospholipid bilayers, since the head group of the lecithin was not modified chemically at all. Moreover, the pyrene moiety will exhibit a relatively long lifetime and high quantum yield of fluorescence emission,[19,22] and, hence, should be suitable for fluorescence studies. High quantum efficiency of the probe allows the use of smaller amounts of the probe, which should reduce the probe's perturbation of the host lipid environment.[18] DPDL has another unique feature worth noting as a probe diagnosing the nature and state of lipid bilayers. DPDL carries a pyrene moiety at the end of each fatty acyl chain of the lipid molecule and will easily adopt a sandwich configuration of the pyrene moieties in the ground state. Hence, it can easily form an intramolecular exciplex from the ground-state dimer[23,25] and will provide useful information concerning the inter- and/or intramolecular interactions between the fatty acid chains located in a very hydrophobic domain. A small probe containing a single fluorescent group, ethyl ω-(1-pyreno)decanoate (EP), was also used for comparison. Using these probes, we have investigated the structure of phospholipid bilayers as well as the location and orientation of the probe molecules in the bilayers.

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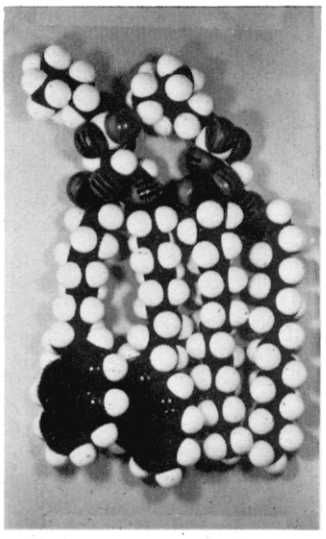

Figure 1. CPK models of the pyrene-labeled lecithin DPDL (left) and dipalmitoylphosphatidylcholine (right).

Materials and Methods

The steps used for the preparation of fluorescent probes are shown in Figure 2. The general route is based on the methods of Chadha[26] and Chakrabarti and Khorana.[20]

Synthesis of Modified Fatty Acid. ω-Carbethoxypelargonoyl Chloride (III). ω-Carbethoxypelargonoic acid (II) was prepared in 82% yield from sebacic acid (216 g, 1.08 mol) and diethyl sebacate (165 g, 0.64 mol) in 60 mL of di-*n*-butyl ether and 88 mL of ethanol containing 30 mL of concentrated hydrochloric acid.[27] II was converted to III in 93% yield by treatment with thionyl chloride in petroleum ether:[28] IR (neat) 1795 (COCl), 1730 cm^{-1} (C=O, ester).

1-(9-Carbethoxy-1-oxononanyl)pyrene (IV). A mixture of III (79 g, 0.33 mol), aluminum chloride (80 g, 0.60 mol), and 100 mL of carbon disulfide was cooled with an ice–salt bath, and 350 mL of pyrene (60 g, 0.30 mol) solution in carbon disulfide was added dropwise keeping the reaction temperature at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then at 25 °C for 6 h. After 300 mL of water was added at 0 °C, the whole mixture was stirred at 25 °C for an additional 5 h. The product was extracted with carbon disulfide, and the extract was dried over sodium sulfate. Evaporation of the solvent gave the crude product. It was purified by recrystallization from methanol: yield 77 g (62%, based on pyrene); mp 46–48 °C; IR (KBr) 1725 (C=O, ester), 1680 (C=O, ketone), 840 cm^{-1} (CH, pyrene); NMR (CCl_4) δ 1.20 (t, 3 H, J = 6 Hz, CH_3CH_2O), 1.29 (br, 8 H, $(CH_2)_4CH_2CH_2C{=}O$), 1.79 (m, 2 H, $-CH_2CH_2C(=O)$ pyrene), 2.18 (t, 2 H, J = 8 Hz, $CH_2C(=O)O$), 3.03 (t, 2 H, J = 8 Hz, $CH_2C(=O)$ pyrene), 4.01 (q, 2 H, J = 6 Hz, $CH_3CH_2OC(=O)$), 8.09 (m, 8 H, pyrene), 8.78 ppm (d, 1 H, $J_{2\text{-}3}$ = 7 Hz, pyrene $H(2)$). Anal. Calcd for $C_{28}H_{30}O_3$: C, 81.13; H, 7.29. Found: C, 81.19; H, 7.26.

10-(1-Pyreno)decanoic Acid (V). A mixture of IV (22 g, 0.05 mol), potassium hydroxide (10.2 g, 0.18 mol), and hydrazine hydrate (8.5 g, 0.17 mol) was dissolved in 210 mL of diethylene glycol and refluxed for 4 h at 162–178 °C. Excess hydrazine hydrate was distilled off at 178–220 °C. After cooling the residue to 25 °C, 250 mL of water was added dropwise followed by stirring for 2 h at room temperature. The reaction mixture was acidified with 800 mL of 6 N hydrochloric acid to afford precipitate. The precipitate was isolated by filtration, washed thoroughly with water, and then dried in vacuo. The crude sample was purified further by column chromatography twice on Wakogel C-100 (1.8 × 60 cm) with chloroform as an eluant. The yield was 86% (16 g): mp 113–114 °C; IR (KBr) 3370 (OH), 1695 (C=O, carboxylic acid), 830 cm^{-1} (CH, pyrene); NMR ($CDCl_3$) δ 1.26 (br, 10 H, pyrene $CH_2CH_2(CH_2)_5$), 1.79 (br, 4 H, pyrene $CH_2CH_2(CH_2)_5CH_2$), 2.29 (t, 2 H, J = 6 Hz, $CH_2C(=O)O$), 3.27 (t, 2 H, J = 8 Hz, pyrene CH_2), 7.92 (m, 9 H, pyrene), 10.30 ppm (s, 1 H, $C(=O)OH$). Anal. Calcd for $C_{26}H_{28}O_2$: C, 83.83; H, 7.58. Found: C, 83.66; H, 7.62.

Ethyl ω-(1-Pyreno)decanoate (EP). The acid chloride of V was prepared by treating V with about a tenfold excess of freshly distilled thionyl chloride at 40–60 °C for 9 h. Removal of the excess thionyl chloride under reduced pressure afforded the corresponding acid chloride: IR (neat) 1785 cm^{-1} (COCl). It was converted to the ethyl ester on treatment with dry ethanol. The crude product was purified three times by column chromatography on Wakogel C-100 (1.8 × 60 cm) with chloroform as an eluant: yield 50%; mp 46–47 °C; IR (KBr) 1725 (C=O), 1170 (C—O—C), 825 cm^{-1} (CH, pyrene). Anal. Calcd for $C_{28}H_{32}O_2$: C, 83.96; H, 8.05. Found: C, 83.74; H, 8.09.

Synthesis of Modified Phospholipids. *sn*-Glycero-3-phosphorylcholine. Egg yolk lecithin (egg L) was isolated and purified from egg yolk.[29,30] *sn*-Glycero-3-phosphorylcholine–cadmium chloride complex was prepared in 55% yield from the purified egg yolk lecithin essentially by the procedures given in the literature:[20,26] mp 118–120 °C; IR 3400 (OH), 1200 (P=O), 1055 cm^{-1} (P—O—C). Anal. Calcd for $C_8H_{20}NO_6P\ CdCl_2{\cdot}H_2O$: C, 20.96; H, 4.84; N, 3.05. Found: C, 20.65; H, 4.62; N, 2.98.

1,2-Bis[ω-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphorylcholine (DPDL). A solution of the acid chloride of V (6.3 g, 16.3 mmol) in anhydrous ethanol-free chloroform (60 mL, freshly distilled) was added dropwise to a dry powder of the cadmium complex of *sn*-glycero-3-phosphorylcholine (1 g, 2.2 mmol), which was kept at 0 °C

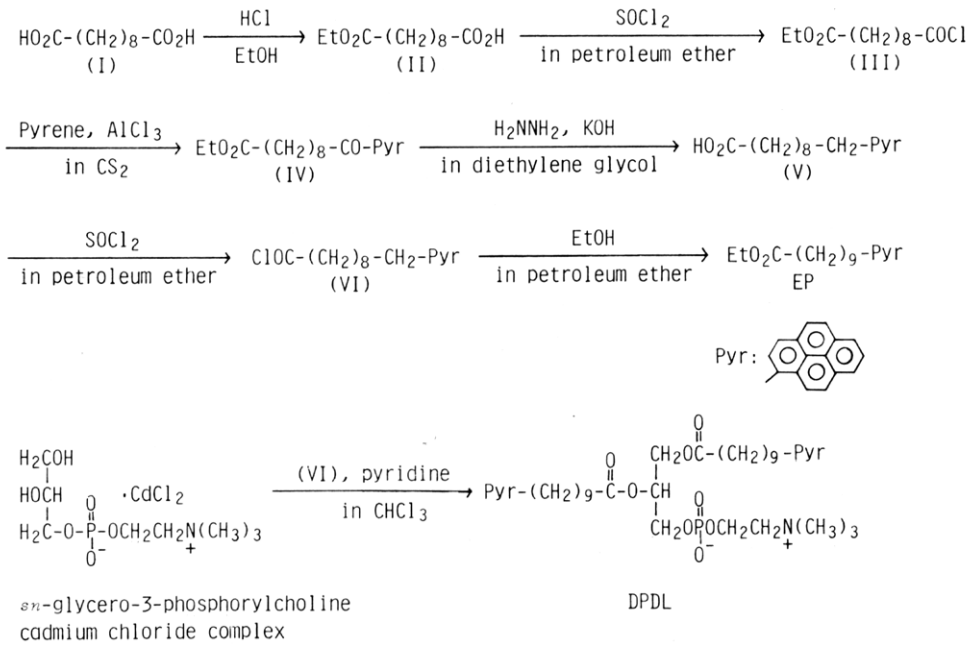

Figure 2. Steps used for the synthesis of the pyrene-labeled lecithin (DPDL) and ethyl ω-(1-pyreno)decanoate (EP).

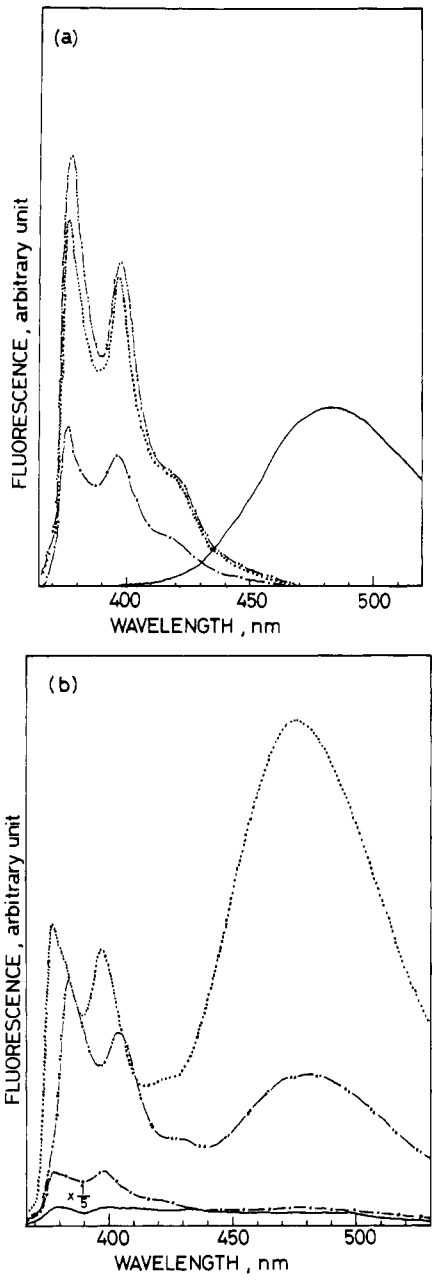

Figure 3. Fluorescence emission spectra of 7.42 μM EP (a) and 13.7 μM DPDL (b) in hexane (— · —), chloroform (- · · -), ethanol (· · ·), and aqueous buffered solution (pH 5) containing 8% (v/v) ethanol (—) at 20 °C.

under dry nitrogen atmosphere. With stirring, anhydrous pyridine (2.6 g, 33 mmol) dissolved in 10 mL of anhydrous chloroform was added slowly over 30 min. The reaction mixture was stirred at 0 °C for 1 h, then at 25 °C for 4 h, and finally refluxed for 1 day. To the mixture was added slowly 50 mL of anhydrous methanol. After evaporation of the solvent in vacuo below 40 °C, the residue was redissolved in 25 mL of chloroform–methanol–water (5:4:1 by volume) and subjected to column chromatography on a mixed ion-exchange resin (Amberlite IR-45 and IRC-50, 1.8 × 70 cm) with the same solvent as an eluant. After evaporation of the solvent, the residue was dissolved in 5 mL of chloroform and passed through a column of silica gel (Wakogel C-100, 1.8 × 70 cm) using chloroform–methanol–water (65:25:4 by volume) as an eluant. The effluent was monitored by TLC and the fractions that contained a spot whose mobility was reasonable for DPDL were pooled and evaporated to dryness. The overall yield from *sn*-glycero-3-phosphorylcholine–cadmium chloride complex was 23%: IR (KBr) 1720 (C=O), 1220 (P=O), 1050 (P—O—C), 825 cm^{-1} (CH, pyrene); NMR ($CDCl_3$) δ 1.69 (s, 24 H, OC(=O)-$CH_2CH_2(CH_2)_6CH_2$ pyrene), 1.98 (br, 4 H, OC(=O)CH_2CH_2), 2.70 (br, 4 H, OC(=O)CH_2), 3.60 (br, 9 H, $N^+(CH_3)_3$), 3.92–3.96 (br, 12 H, $CH_2OC(O)$ and $POCH_2CH_2N^+$), 5.40 (br, 1 H, CHO), 7.86 ppm (m, 18 H, pyrene). Although the molecular ion peak was not observed in the mass spectrum, the following fragment ion peaks were observed (peaks whose intensity was greater than 1% of the base peak are presented here): *m/e* 523, 373, 327, 217, 169, 98, 73, 57 (base peak). This fragmentation pattern was in good agreement with the previous findings of Perkins and Johnston[31] and Klein[32] for a number of glycerophosphorylcholines. The molecular ion peak was not detected in their investigations either.

Other Lecithins. Synthetic dipalmitoyl-DL-α-phosphatidylcholine (DPL) was used as received from Sigma Chemical Co. Egg yolk lecithin (egg L) was isolated and purified from egg yolk as described above.

The purity of all the lecithins used, DPDL, DPL, and egg L, was also established by TLC on silica gel plates (Tokyo Kasei Kogyo Co., Tokyo) in chloroform–methanol–water (65:25:4 by volume). Only one spot corresponding to the phosphatidylcholine was revealed when detected by UV light, iodine staining, and Dragendorff's reagent.

Preparation of Liposomes. Multicompartment liposomes containing fluorescent probes were prepared by the injection technique.[33,34] Thus, 300 μL of ethanolic solution of lecithin (38 mg in 5 mL of ethanol) was injected rapidly into 10 mL of a magnetically stirred buffer solution (0.28 M acetate containing 0.1 M NaCl, pH 5). During injection the temperature was kept at about 10 °C higher than the phase-transition temperature of the phospholipid employed. In the case of liposomes containing cholesterol and/or fluorescent probes in the liposomal bilayer, an ethanolic solution containing lecithin, probe, and/or cholesterol at an appropriate molar ratio was injected. The liposome solution prepared in this way was clear enough for the fluorescence measurement. The formation of liposomes was visualized as negatively stained with 2% aqueous sodium phosphotungstate in acetate buffer (pH 4) on a JEOL JEM-100 V electron microscope. They were relatively homogeneous multicompartment liposomes of less than 50 nm in diameter.

Fluorescence Measurements. All the solvents used were carefully purified, dried, and stored over molecular sieves Linde $4A_{1/16}$ or $3A_{1/16}$. Water contents in these solvents were determined each time before use on a Karl-Fisher Moisture Automatic Titration Apparatus Model MK-A II (Kyoto Electronics MFG. Co., Ltd., Kyoto). They were less than 6 ppm in chloroform, 5 ppm in hexane, 7 ppm in benzene, and 93 ppm in ethanol. All fluorescence measurements of DPDL and EP were made in the subtraction mode on a Hitachi 512 spectrofluorometer with excitation at 341.5 nm and slit widths of 10 and 3 nm for excitation and emission sides, respectively. Measurements as a function of temperature were made in a thermoregulated cell compartment equipped with a Komatsu-Yamato Coolnics Model CTR-120. An average cooling or heating rate of 9–10 °C/h was used during the temperature scan over a range of 15–48 °C. That the temperature was effectively constant during the time needed to record a fluorescence spectrum was ascertained beforehand.

In order to avoid the formation of ground-state aggregates of the probe within the lecithin bilayers, the molar ratio of phosphatidylcholine to DPDL or EP was always kept in the range of 500–1000 to 1.[9] This should also help to minimize the impurity effect of the probes on the phase transition of lecithins.[18]

All the fluorescence measurements were made under atmospheric conditions. No degassing was carried out since we could not attain a good reproducibility of the data on degassed samples of liposome solutions because of extreme instability of liposome solutions during degassing procedures on a vacuum line at liquid nitrogen temperature. Bubbling gaseous nitrogen into liposome solutions also gave unreliable data. Under atmospheric conditions, however, good reproducibility was attained for both cases in liposome solutions and in organic solvents. Since the fluorescence intensity is proportional to the quantum yield when the exciting light intensity and the concentration of absorbing molecules are kept constant, the fluorescence intensities for the monomer, I_m (378 nm), and excimer, I_e (480 nm), were obtained from the normalized spectra.

Results and Discussion

Electronic Absorption Spectra. The absorption spectra of DPDL and EP in the organic media employed were found to obey Lambert–Beer's law at all wavelengths examined in the concentration range of 1–20 μM. In aqueous buffered solutions

Table I. Relative Intensities of Monomer and Excimer Emissions of DPDL and EP in Different Media at 19 °C[a]

in hexane			in chloroform			in ethanol		
	I_e	I_m		I_e	I_m		I_e	I_m
[EP], μM			[EP], μM			[EP], μM		
1.98		20	1.98		76	1.98		45
3.95		36	3.95		112	3.95		85
5.92		48	5.92		155	5.92		122.5
7.88		64	7.88		195	7.88		150
9.83		85	9.83		227.5			
11.78		95	11.78		255			
[DPDL], μM			[DPDL], μM			[DPDL], μM		
7.62	17.5	55	7.62	150	245	2.56	132.5	80
10.13	22.5	65	10.13	262.5	360	5.10	327.5	192.5
12.62	30	82.5	12.62	257.5	495	7.62	612.5	365
15.10	37.5	95	15.10	360	615	10.13	635	370
17.56	55	112.5						

[a] Relative intensities, I_e and I_m, are readings on the recorder calculated from normalized fluorescence spectra.

also they obeyed Beer's law at least in the low concentration range (1–7.5 μM for EP and 1–6 μM for DPDL). Because of their poor solubilities in water, higher concentrations could not be examined. Electron micrographic studies indicated that DPDL forms bilayers in aqueous media just as DPL or egg L does, while EP seemed to form micelles or aggregates under identical conditions. In any event, the resulting aqueous buffered DPDL solution containing 8% (v/v) ethanol was transparent enough to allow optical measurements. In organic solvents over the concentration range examined, no other absorption bands were found at a longer wavelength for either probe.

Fluorescence in Solutions. Figure 3 illustrates the fluorescence emission spectra of EP (a) and DPDL (b) in different media under the same conditions. EP shows only monomer emission bands of fluorescence at around 377 and 398 nm in organic solvents, while in aqueous buffered solutions only fluorescence emission from the excimer of pyrene moiety was observed at 480 nm.[19,22] In organic solvents, the intensity of monomer emission increases with the stoichiometric concentration of EP (Table I). Within the concentration range examined (1–12 μM), no excimer emission from EP was observed. On the other hand, DPDL shows both monomer and excimer emission bands of fluorescence in all the media examined even at the relatively low concentration (2.5 μM). Relative intensities of both emissions increased as a linear function of the stoichiometric concentration of DPDL (Table I). In aqueous buffered solution, the fluorescence emission from DPDL was quenched to a great extent, but still both the monomer and excimer emissions were definitely observable.

Excimer Formation. As illustrated in Figure 4, the I_e/I_m value of DPDL in a given solvent is nearly constant over the concentration range examined. An interesting fact emerging from the data shown in Figure 4 is the enhanced value of the relative quantum efficiency in ethanol as compared with those in hexane or chloroform (1.7 vs. 0.4–0.6). The corresponding relative quantum efficiency of pyrene is not dependent so much on the solvent polarity.[22,35,36] The corresponding values for dodecylammonium pyrene-1-butyrate (DAPB) (2.58 in benzene and 0.83 in ethanol[22]) were completely reversed to those observed for DPDL in this work. This was explained in terms of the favorable self-association of DAPB in benzene leading to the increase in the effective concentration of pyrene aggregates.[22]

Since the two pyrene moieties of DPDL can intramolecularly adopt a sandwich configuration (see CPK model in Figure 1), intramolecular dimer formation occurs easily in the ground state.[23-25] Moreover, at sufficiently high concentrations of the probe an intermolecular aggregate is also formed in the ground

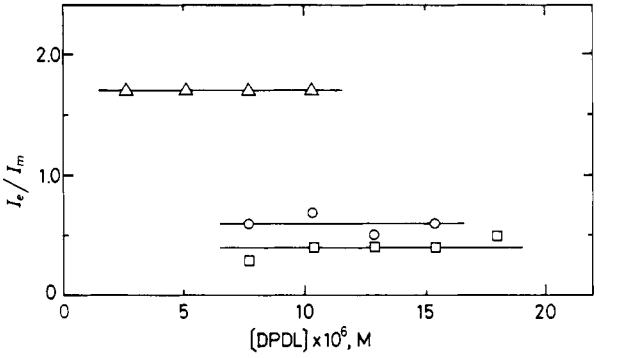

Figure 4. Relative fluorescence quantum efficiencies I_e/I_m for DPDL in ethanol (△), chloroform (○), and hexane (□) as a function of probe concentration.

state. As is often the case the formation of ground-state dimers or higher aggregates will induce a shift in the spectrum.[37] This is verified in the absorption and fluorescence spectra of DPDL in aqueous media. It shows a new absorption band at 357 nm, and the fluorescence emissions are drastically quenched, since the aggregate is nonfluorescent and absorbs a portion of light[37] (see Figure 3b). If the excimer was brought about by ground-state intermolecular aggregation, the equilibrium formation of the excimer must be observed since some of the excited molecules are altered resulting in a decrease in the quantum yield of the monomer population.[22,35,36,38,39] However, in the present case the compensation in intensities between monomer and excimer emissions of DPDL as a function of stoichiometric concentration was not observed (Table I and Figure 4). This is good evidence for the formation of an intramolecular excimer.[40]

The solvent polarity dependencies of the relative quantum efficiency, I_e/I_m, can be interpreted in terms of two distinct configurations of fatty acid residues of DPDL (Figure 5).[41] If DPDL exists in the solvent-separated configuration, the quantum yield of monomer emission must be predominant. On the other hand, the monomer emission of fluorescence will not be expected from the solvent-surrounded configuration. In ethanol the equilibrium must trend toward the solvent-surrounded configuration because of favorable hydrophobic and π–π interactions of intramolecular pyrene moieties in polar media. If this is the case, the position of equilibrium in the configuration would be affected only by the solvent polarity, not by the probe concentration. The experimental data were completely consistent with this explanation (Figure 4). In any event, these specific phenomena were characteristic only of DPDL, which contains two fluorophores in the molecule. Very

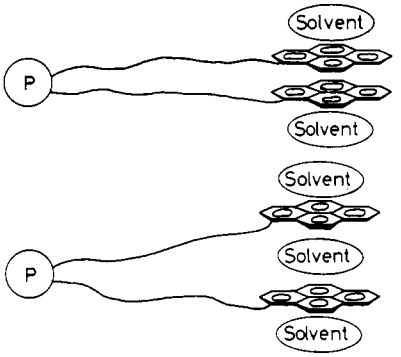

Figure 5. Schematic representation of two distinct configurations of DPDL in solution: the solvent-surrounded (top) and solvent-separated configurations (bottom) of the pyrene-labeled fatty acyl chains.

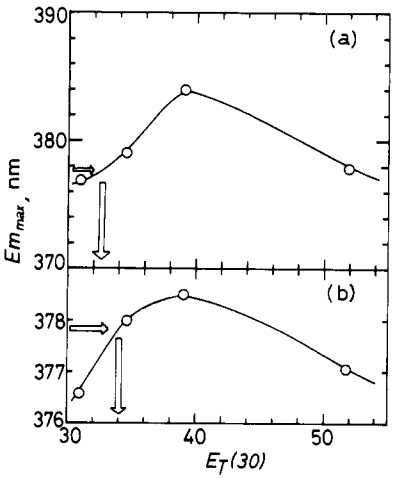

Figure 6. Plots of fluorescence emission maxima of DPDL (a) and EP (b) in different solvents against solvent polarity parameter $E_T(30)$. From left to right, in hexane ($E_T(30)$ = 30.9), benzene (34.5), chloroform (39.1), and ethanol (51.9). A blank arrow indicates the polarity of microenvironment around probes intercalated in liposomal bilayers.

recently, Morawetz and his co-workers demonstrated similar results in studies on intramolecular excimer formation using bis(1-arylmethyl) ethers, where the naphthyl and biphenyl compounds yielded the excimer more significantly in ethanol than in heptane.[40]

Microenvironment of Probes in Liposomal Bilayers. A knowledge of the location of probes in liposomes is required before the studies on the permeation of probes into the liposomal bilayers. The microenvironment around probes in liposomal bilayers has been estimated by correlating fluorescence maxima of the probes with the microscopic solvent polarity parameter, Dimroth's $E_T(30)$.[13,14,43] Figure 6 includes such plots of emission maxima of fluorescent monomer against $E_T(30)$. The data suggest that the apparent environment of DPDL and EP in liposomal bilayers is roughly similar to that in carbon tetrachloride ($E_T(30)$ = 33.9) or toluene (32.5). Though some ambiguities remain in the reference scale itself since the emission maxima of probes in ethanol shift to a shorter wavelength (Figure 6), a similar observation has been reported for another fluorescent probe, dansylated octadecylamine, by Romero et al.[14] Judging from their intrinsic hydrophobic nature, it is tempting to believe that these probes are located in a very hydrophobic domain of the liposomal membranes. The same conclusion was reached also from the correlation of the relative intensities of monomer emission with Dimroth's $E_T(30)$ (data not shown).

Codispersion of Probes with Phospholipids into Aqueous Buffered Solutions. The fluorescence spectral change of DPDL

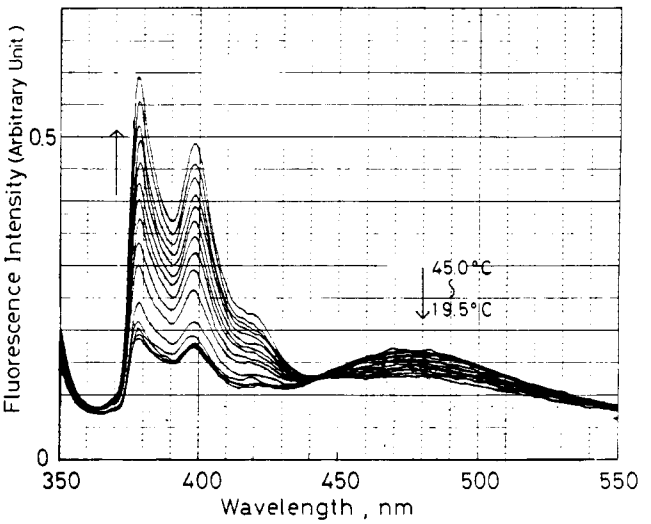

Figure 7. Fluorescence spectra of DPDL as a function of temperature in the dipalmitoylphosphatidylcholine (DPL) bilayers without cholesterol. DPDL (0.471 μM) was codispersed with 0.312 mM DPL into 0.28 M aqueous acetate buffer containing 0.1 M NaCl at 50 °C.

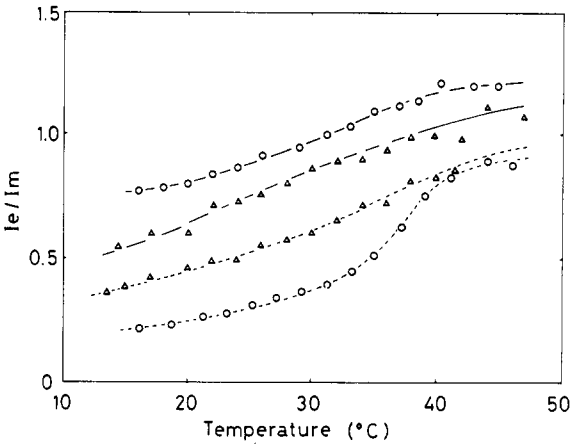

Figure 8. The relative quantum efficiencies, I_e/I_m, of DPDL in the DPL (○) and egg L (△) bilayers as a function of temperature in the absence (broken line) or presence (solid line) of cholesterol (38.9 mol % for egg L and 39.3 mol % for DPL). DPDL (0.471 μM) was codispersed with lecithin (0.312 mM) above chain-melting transition. The solution was slowly cooled down. The second heating curve coincides with the first cooling curve. Conditions: 0.28 M acetate buffer containing 0.1 M NaCl; see text for details.

in the dipalmitoylphosphatidylcholine bilayers without cholesterol is shown in Figure 7 as a function of temperature. Under these conditions there exists a well-defined isolampsic (isoemissive) point at 445 nm. The relative quantum efficiencies of DPDL in two different bilayers (DPL and egg L) with or without cholesterol are plotted against temperature in Figure 8. The presence of cholesterol made the relative quantum efficiency larger in both egg and dipalmitoylphosphatidylcholine liposomes. Its effect was more prominent below the phase-transition temperature of dipalmitoylphosphatidylcholine bilayers. In addition, the presence of cholesterol abolished the phase transition of the lipid resulting in an increase in the mobility of fatty acid chains and disruption of intermolecular interactions between fatty acid chains.[44-47] Increasing temperature had a similar effect on the I_e/I_m ratio.

Above the phase-transition temperatures, the lateral diffusion of lipids occurs in bilayers,[1,5,12,21,48] resulting in the conformational change of the probe in bilayers with a concomitant change in the I_e/I_m ratio. It must be noted here that

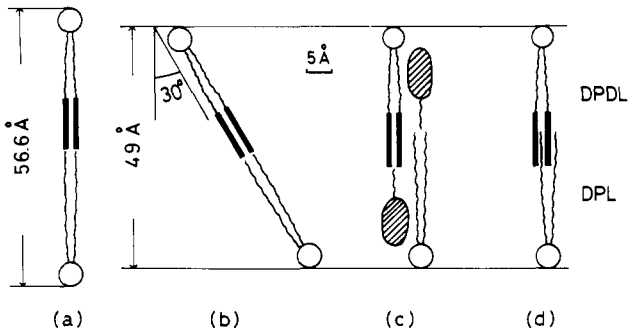

Figure 9. Schematic representation of the structure of the liposomal bilayers. The open circle represents the polar head groups of the phospholipid molecules. The curved lines represent the acyl chains. The blocked group represents the pyrene moiety of DPDL. The hatched group represents the steroid nucleus of cholesterol. (a) Fully extended structure. (b) The tilted conformation with respect to the bilayer plane to hold the thickness (49 Å) with fully extended hydrocarbon chains (L_β state). (c) The shrunk structure with the lipid-intimate conformation, where cholesterol fills the space to fit best. (d) The shrunk structure with the lipid-separated conformation.

all bilayers were formed at a higher temperature than the phase-transition temperature of lecithins adopted, and the relative quantum efficiency was monitored by cooling down from the chain-melting transition temperature. The curves obtained on repeat of heating were superimposable to the first cooling curve. Unlike DPDL, EP did not show any excimer formation in the lipid bilayers of DPL or egg L under the identical conditions.

From the results stated above, the following points are now clear. (1) The relative quantum efficiency is larger in the softer egg phosphatidylcholine bilayers than in the more rigid bilayers of dipalmitoylphosphatidylcholine. (2) The addition of cholesterol makes the I_e/I_m values larger. (3) Increasing temperature also caused an increase in the I_e/I_m ratio. (4) In the case of dipalmitoylphosphatidylcholine bilayers without cholesterol, the relative quantum efficiency drastically increases at the pretransition temperature (30 °C) and levels off above the chain-melting transition temperature (41 °C).[4] (5) The observed I_e/I_m-temperature profile is thermotropically reversible. These results strongly suggest to us that a temperature-dependent equilibrium associated with a conformational change of the liposomal bilayers has been observed.

Conformation of Probes in Liposomal Bilayers. If the hydrocarbon chains of dipalmitoylphosphatidylcholine take a fully extended conformation, as demonstrated previously,[4] the thickness of the bilayer should be about 56.6 Å. However, careful electron micrographic investigation showed that the thickness of the bilayer is always less than 50 Å. Our observation is not inconsistent with those obtained by the sedimentation technique[49] and X-ray diffraction studies on phosphatidylcholine liposomes.[50] In order to match the observed thickness, the structure of bilayers must be either the tilted conformation (Figure 9b), or the shrunk one (Figure 9c or d). In the former model,[4] a structural transformation of bilayers takes place at the pretransition from a one-dimensional lamellar lattice with crystalline-like chains tilted with respect to the bilayer plane, L_β (Figure 9b), to a two-dimensional monoclinic lattice with crystalline-like chains perpendicular to the bilayer plane, P_β. This is followed by a second transformation to a one-dimensional lamellar lattice with a hydrocarbon chain conformation in a liquid paraffin-like arrangement, L_α, above the chain-melting transition temperature.[4,48] Interestingly, the observed I_e/I_m-temperature profile for the dipalmitoylphosphatidylcholine bilayers without cholesterol (Figure 7) was consistent with the data obtained by DSC and low-angle X-ray diffraction studies.[4] However, the CPK model suggests that even when DPDL was tilted by 30–35° with respect to the bilayer plane, the pyrene moieties still can adopt a sandwich configuration in the bilayers, which would result in the formation of more excimer at low temperatures. This is apparently inconsistent with the results obtained here.

Another possibility for the bilayer structure is the lipid-separated conformation (Figure 9d). In the deepest region of bilayers, the fatty acid chains of opposite layer may play a role just as the nonpolar solvent molecules do in solution. If this is the case, DPDL should take the lipid-separated conformation in the bilayers without cholesterol and/or below the phase-transition temperature. Under these circumstances, the intramolecular ground-state dimer must be disrupted to result in the low relative quantum efficiency. Increasing temperature and/or penetrating cholesterol increase the mobility of lipid and probe molecules in the hydrophobic domain,[1,6,12,21,51] which increases the probability that more ground-state dimer will be formed with a concomitant increase in the I_e/I_m ratio. These results are consistent with those obtained from the dynamic studies on permeations of DPDL and EP into the liposomal bilayers, which will be described elsewhere.

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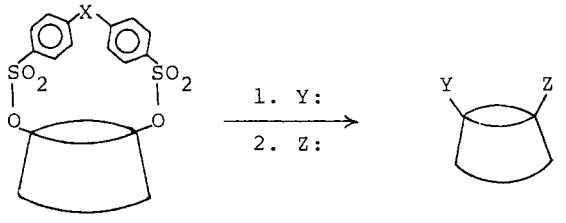
Communications to the Editor

The First Successful Carbonic Anhydrase Model Prepared through a New Route to Regiospecifically Bifunctionalized Cyclodextrin

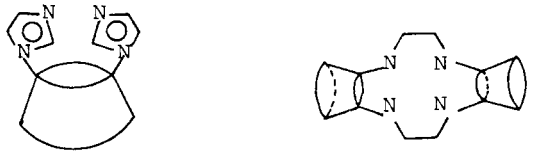
Sir:

Cyclodextrins or their simple derivatives have now become very common as enzyme models in biomimetic chemistry.[1] For the construction of more refined and sophisticated enzyme models, however, the regiospecific multifunctionalization of cyclodextrins has become increasingly important since the convenient *capped cyclodextrin route*,[2,3] from **1** to **2**, has been developed,[3,4] as exemplified by a successful nuclease model[3] involving the use of bis(*N*-imidazolyl)cyclodextrin (**3**) or in the preparation of a promising multirecognition molecule,[5] duplex cyclodextrin (**4**). However, the attempted bifunction-



1a X = CH_2
b X = O

2



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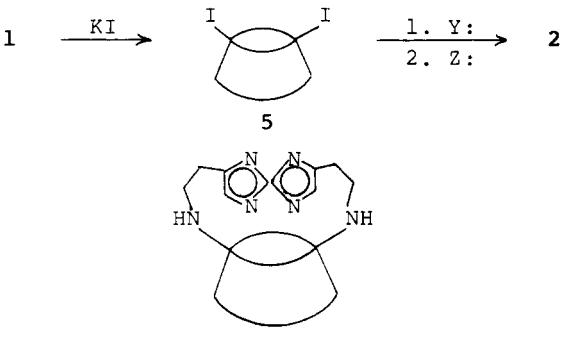
alization of cyclodextrin directly from **1** through the replacement by weak nucleophile(s) often encounters difficulties because of the insufficient reactivity of **1** and, therefore, a new preparative route should be developed.

The authors report now a new and much more facile route to bifunctionalized cyclodextrins from **1a** through the corresponding diiodide (**5**) and its successful application to the preparation of the first *carbonic anhydrase model*, **6** and **3**, the former of which especially affords reasonable activity. Thus, **1a** (6.3 mmol) was treated with potassium iodide (180 mmol) in 300 mL of dry DMF at 80 °C for 2 h. After the usual



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workup, followed by the reprecipitation from water, the addition of tetrachloroethylene gave **5** in 95% yield. The structure of **5** was ascertained by its conversion into known disubstituted cyclodextrins including di(ω-aminoethylamino)-β-cyclodextrin[6] or di(*N*-imidazolyl)-β-cyclodextrin.[3] NMR and IR spectra of **5** were also satisfactory.

The diiodide, **5**, treated with (an) appropriate nucleophile(s) gave a disubstituted cyclodextrin, **3** or **5**, very readily. Thus, **5** was treated with 10 molar excess of histamine in DMF at 45 °C for 4 h to give bis(*N*-histamino)-β-cyclodextrin (**6**) in 34% yield. After the reaction mixture cooled, the resultant precipitate was collected by filtration and dissolved in water. The addition of tetrachloroethylene gave a precipitate which was further purified by the preparative paper chromatography: IR 1550, 1480 cm^{-1} and other cyclodextrin absorptions; NMR δ 2.90 (8 H), centered at 3.75 (42 H), 5.05 (7 H), 6.82 (2 H), 7.57 (2 H). Bis(*N*-imidazolyl)-β-cyclodextrin (**3**) was similarly prepared by reaction of **5** with imidazole at 80 °C for 4 h in 46% yield.[7]

Carbonic anhydrase has Zn^{2+} surrounded by three imidazoles in its active site and CO_2 is bound to the active site in close proximity[8] to Zn^{2+} with assistance of hydrophobic interactions.[9] Water activated by Zn^{2+} coordination[10-12] attacks CO_2 bound where the base catalysis by an imidazole may be involved.[12] However, the detailed mechanism is still uncertain, and no appropriate model study has been carried out.[13,14] Our present compound, **6** or **3**, affords a hydrophobic pocket, Zn^{2+} bound to imidazoles located at the edge of the pocket, and also additional bases in the case of **6**, providing an appropriate model of carbonic anhydrase.

A thermostated solution of either enzyme model **3** or **6** was mixed with a thermostated solution of CO_2 by use of a stopped-flow spectrophotometer, and the formation of car-

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