Design, Synthesis, and Fluorescence Studies of Fluorenyl Fatty Acids as New Depth-Dependent Fluorescent Probes for Membranes: Getting over the Looping-Back Problem†

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ABSTRACT: Fluorescent fatty acids have proved very useful in studying the membrane hydrophobic core. They readily partition into membranes or can be converted to phospholipids, which form integral components of membranes. By attaching the fluorescent chromophore to different positions along the alkyl chain of fatty acids, e.g., an anthroyloxy group attached via an ester linkage to *n*-hydroxystearic acid, membranes have been probed at different depths. While this is an interesting approach and has been extensively used, relatively little attention has been paid to the molecular design of these probes in order to have minimal membrane perturbation. In the present study we have looked into the general problem of design of such depth-dependent membrane probes. We report here a series of fluorenyl fatty acids with varying fatty acid chain lengths, i.e., (2-fluorenyl)acetic acid, -butyric acid, -hexanoic acid, and -octanoic acid, in order to obtain information at different depths in the membrane hydrophobic core. To see the effect of attachment of a hydrophobic tail on the orientation of such fatty acids in membranes, an *n*-butyl group was linked to the C-7 position of fluorene in (2-fluorenyl)butyric acid to get 4-(7-*n*-butylfluoren-2-yl)butyric acid. Further, to assess their ability to act as depth-dependent fluorescent probes, these fatty acids were incorporated in vesicles prepared from egg phosphatidylcholine, and their fluorescence quenching was studied with potassium iodide, Cu(II), 9,10-dibromostearic acid, and 12-bromostearic acid. The Stern–Volmer plots so obtained clearly indicated the following depth order: 8-(2-fluorenyl)octanoic acid > 6-(2-fluorenyl)hexanoic acid > 4-(7-*n*-butylfluoren-2-yl)butyric acid > 4-(2-fluorenyl)butyric acid > (2-fluorenyl)acetic acid. These results indicate that the fluorenyl fatty acids reported here do probe the membrane at different depths, depending on their transverse location. In addition, the attachment of a hydrophobic tail helps in better aligning the probe in membranes and could thus overcome the problems associated with looping back of such fatty acids to the membrane–water interface. These results were also supported by fluorescence polarization data. The general design strategy utilized here can in principle be extended to other membrane probes like photoactivatable fatty acids.

Fluorescent probes have been widely used to study membrane structure and dynamics (Azzi, 1975). Fatty acids that bear a fluorescent chromophore have proved very useful as such or on subsequent transformation to phospholipids (Lakowicz, 1981; Thulborn, 1981; Vincent & Gallay, 1984; Sunamoto et al., 1980). However, like other probes these probes have also been criticized for possible perturbation caused by them on incorporation into membranes. This argument has been specially forwarded in the case of anthroyloxy fatty acids (Thulborn & Sawyer, 1978; Kuroda et al., 1986) wherein the fluorescent group protrudes as a pendent group from the fatty acyl chain. More recently, pyrene- (Waka et al., 1980; Jones & Lentz, 1986) and anthracene- (de Bony & Tocanne, 1983; Vincent et al., 1985; Molotkovsky et al., 1984) based fatty acids have been reported and used as fluorescent probes. These fatty acids contain a pyrene or anthracene chromophore linked to the ω-carbon atom.

The main objective of this study was to look into the design of fluorescent fatty acids in order to obtain depth-dependent probes that are oriented in membranes like normal fatty acids and cause minimal membrane perturbation. In this context we report here the synthesis of fluorene-based fatty acids of varying lengths so that the membrane environment at different depths can be monitored. A hydrophobic tail has been attached to one of these fluorenyl fatty acids to enable improved hydrophobic interaction and proper alignment in membranes. These fluorenyl fatty acids have been incorporated in PC[1] vesicles, and their orientation in membranes has been studied by fluorescence quenching with extrinsic, e.g., iodide, and intrinsic, e.g., 12-bromostearic acid, quenchers. The depth-dependent quenching data clearly indicate that the attachment of a hydrophobic tail assists in better alignment of the fluorescent probes in membranes. This approach in principle can be extended to existing fluorescent as well as photoactivable fatty acids, resulting in more effective design for probing membranes at different depths.

Materials and Methods

Chemical Synthesis. All chemicals and solvents were commercial grades of highest purity available and were further purified, if required, according to Perrin et al. (1980). UV–visible spectra were recorded on a Shimadzu UV-190 or UV-260 spectrometer. NMR spectra were recorded on a Varian XL-100 or a Bruker 500-MHz spectrometer. Mass

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[1] Abbreviations: TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; C2A-FL, (2-fluorenyl)acetic acid; C4A-FL, 4-(2-fluorenyl)butyric acid; C6A-FL, 6-(2-fluorenyl)hexanoic acid; C8A-FL, 8-(2-fluorenyl)octanoic acid; C4A-FL-C4, 4-(7-*n*-butylfluoren-2-yl)butyric acid; PC, phosphatidylcholine.

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spectra were recorded on a Varian MAT-112 S or a Shimadzu QP-1000 spectrometer. HPLC analysis was carried out on a Du Pont 8800 system or a Shimadzu LC-4A system using a Zorbax amine column. The mobile phase used was acetonitrile–water (88:12) at a flow rate of 1 mL/min. Samples were monitored at 260 nm by using a Shimadzu SPD-2A UV detector. All HPLC analyses were carried out under these conditions unless specified otherwise. (2-Fluorenyl)acetic acid (I, C2A-FL), mp 184–185 °C, was prepared by acylating fluorene with ethyl oxalyl chloride followed by Wolff–Kishner reduction according to the procedure of Stiller et al. (1972).

(2-Fluorenyl)butyric Acid (II, C4A-FL). Fluorene (7 g, 0.042 mol) was dissolved in nitrobenzene (40 mL), and succinic anhydride (4.63 g, 0.046 mol) was added to it. The reaction mixture was stirred for 15 min with cooling at 0 °C. Anhydrous aluminium chloride (11.21 g, 0.084 mol) was then added in portions with constant stirring. The reaction mixture was further stirred for 1 h at 0 °C and then kept at 4 °C for 36 h. The reaction mixture was then poured on crushed ice and acidified with dilute hydrochloric acid. Subsequently, nitrobenzene was steam distilled. The crude product was dissolved in 5% NaOH and regenerated by acidification. It was then crystallized from acetic acid to give 4-(2-fluorenyl)-3-oxobutyric acid in 93% yield (10.5 g): HPLC R_t 6.2 min; mp 205–206 °C; IR 1720 cm^{-1} (acid carbonyl), 1680 cm^{-1} (aryl carbonyl).

The above-mentioned compound (17 g, 0.063 mol) was dissolved in dry ethylene glycol (70 mL). Hydrazine hydrate (10 mL, 0.2 mol) and KOH (10.73 g, 0.19 mol) were added to it, and the reaction mixture was heated at 120–140 °C for 2 h. Excess of hydrazine hydrate and water formed in the reaction was distilled off, and the reaction mixture was refluxed for 5 h. After cooling, it was poured in water and neutralized with dilute hydrochloric acid. The precipitated solid was filtered and dried. TLC analysis using 10% MeOH in $CHCl_3$ as solvent system, indicated that it was a mixture of two components (R_f 0.38, 0.62). This mixture was separated by column chromatography using silicic acid (425 g). The desired acid (II, C4A-FL) was eluted with 20% and 30% ethyl acetate in benzene to obtain 9.9 g of solid material, which was crystallized first from acetic acid and then from benzene–petroleum ether. The final isolated yield was 58%: HPLC R_t 4.4 min; mp 146 °C; IR 1700 cm^{-1} (acid carbonyl); NMR ($CDCl_3$) δ 2.00 (p, 2 H, J = 7 Hz, $HOOCCH_2CH_2CH_2^-$), 2.38 (t, 2 H, J = 7 Hz, $HOOCCH_2CH_2CH_2^-$), 2.73 (t, 2 H, J = 7 Hz, $HOOCCH_2CH_2CH_2^-$), 3.86 (s, 2 H, C_9–H of fluorene), 7.17–7.75 (m, 7 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 252 with base peak appearing at 179 corresponding to fluorenyl–$CH_2^{\bullet+}$.

(2-Fluorenyl)hexanoic Acid (III, C6A-FL). Dry fluorene (15 g, 0.09 mol) was dissolved in dry carbon disulfide (125 mL) and stirred. Anhydrous aluminium chloride (24.1 g, 0.18 mol) was then added in portions. Freshly prepared ethyl adipoyl chloride (22 g, 0.114 mol) in dry carbon disulfide (25 mL) was added dropwise over a period of 1 h and then refluxed for the next 6 h. The excess of carbon disulfide was decanted, and the reaction mixture was washed with more carbon disulfide and petroleum ether. The aluminium chloride complex was then hydrolyzed by dumping the reaction mixture into ice-cold dilute hydrochloric acid. It was stirred at room temperature for the next 4 h to ensure complete hydrolysis. It was extracted with chloroform. The chloroform layer was washed with 10% sodium bicarbonate solution, water, and then with saturated solution of sodium chloride. It was dried over anhydrous sodium sulfate. Excess of chloroform was distilled off on a water bath, and crude material so obtained was subjected to column chromatography over silicic acid (450 g) and eluted out in 15% and 20% ethyl acetate in petroleum ether to give a crude material, which was crystallized from methanol to obtain 6-(2-fluorenyl)-5-oxohexanoic acid ethyl ester (9 g) in 33% yield: HPLC R_t 1.8 min; mp 108–109 °C; IR 1740 cm^{-1} (ester carbonyl), 1690 cm^{-1} (aryl carbonyl); NMR ($CDCl_3$) δ 1.25 (t, 3 H, J = 6.5 Hz, $-COOCH_2CH_3$), 1.6–1.9 (m, 4 H, $-COCH_2CH_2CH_2CH_2COOCH_2CH_3$), 2.4 (t, 2 H, J = 7 Hz, $-COCH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 3.1 (t, 2 H, J = 7 Hz, $-COCH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 4.15 (q, 2 H, J = 6.5 Hz, $-COOCH_2CH_3$), 3.98 (s, 2 H, C_9–H of fluorene) 7.25–8.2 (m, 7 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 322 with the base peak appearing at 193 corresponding to fluorenyl acylium ion.

6-(2-Fluorenyl)-5-oxohexanoic acid ethyl ester (6 g, 0.019 mol) was dissolved in toluene (250 mL) and added to a 1-L round-bottom flask containing zinc amalgam (24 g, 1:4 molar ratio, w/w) in concentrated hydrochloric acid (100 mL). The reaction mixture was refluxed for 8 h when another lot of hydrochloric acid (120 mL) was added. Both the layers were separated, and the aqueous layer was extracted with ether. Both the ether and toluene layers were mixed, washed with water and then a saturated solution of sodium chloride, and finally dried over anhydrous sodium sulfate. Excess of ether was distilled on the water bath, and the toluene was removed by vacuum distillation. The residue so obtained was crystallized from methanol to give pure 6-(2-fluorenyl)hexanoic acid ethyl ester (4 g) in 70% yield; HPLC R_t 4.0 min; mp 70–71 °C; IR 1740 cm^{-1} (ester carbonyl); NMR ($CDCl_3$) δ 1.25 (t, 3 H, J = 7 Hz, $-COOCH_2CH_3$), 1.5–1.8 (m, 6 H, $-CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 2.3 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 2.66 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 3.9 (s, 2 H, C_9-H of fluorene), 4.17 (q, 2 H, J = 7 Hz, $-COOCH_2CH_3$), 7–8 (m, 7 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 308 with base peak appearing at 179 corresponding to fluorenyl–$CH_2^{\bullet+}$.

6-(2-Fluorenyl)hexanoic acid ethyl ester (5.2 g, 0.016 mol) was dissolved in 10% methanolic potassium hydroxide (150 mL), and the reaction mixture was refluxed for 6 h. The reaction mixture was then concentrated to 70–75 mL of its volume and dumped in water, followed by the neutralization with hydrochloric acid. The precipitated material was filtered and dried under vacuum. The residue was crystallized from benzene–petroleum ether mixture to give pure (2-fluorenyl)-hexanoic acid (4.40 g) in 93% yield; HPLC R_t 3.6 min; mp 103–104 °C; IR 1700 cm^{-1} (acid carbonyl); NMR ($CDCl_3$) δ 1.41 (p, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2COOH$), 1.68 (p, 4 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2COOH$), 2.34 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2COOH$), 2.68 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2COOH$), 3.87 (s, 2 H, C_9-H of fluorene), 7–8 (m, 7 H, aromatic protons). Mass spectrum gave the molecular ion peak at m/z 280 with the base peak appearing at 179 corresponding to fluorenyl–$CH_2^{\bullet+}$. Anal. Calcd for $C_{19}H_{20}O_2$: C, 81.42; H, 7.14. Found: C, 81.32; H, 6.81.

(2-Fluorenyl)octanoic Acid (IV, C8A-FL). Dry fluorene (6 g, 0.036 mol) was dissolved in dry carbon disulfide (50 mL) and stirred. Anhydrous aluminium chloride (9.62 g, 0.072 mol) was added in portions. Freshly prepared ethyl suberoyl chloride (10 g, 0.043 mol) in dry carbon disulfide (15 mL) was added dropwise over a period of 1 h and then refluxed for the next 6 h. The excess of carbon disulfide was distilled off,

and the reaction mixture was washed with carbon disulfide (30 mL) and petroleum ether (50 mL). The aluminium chloride complex was then hydrolyzed by dumping the reaction mixture into ice-cold dilute hydrochloric acid. It was stirred at room temperature for the next 4 h to ensure complete hydrolysis. It was extracted with chloroform and washed with 10% sodium bicarbonate solution, water, and finally saturated solution of sodium chloride. It was then dried over anhydrous sodium sulfate and filtered. Chloroform was then distilled off, and the crude material so obtained was subjected to column chromatography over silicic acid (450 g). The desired acylated product was eluted out in 75% chloroform in petroleum ether and was crystallized from methanol to obtain pure 8-(2-fluorenyl)-7-oxooctanoic acid ethyl ester (3.6 g) in 33% yield; mp 90–91 °C; IR 1740 cm^{-1} (ester carbonyl), 1685 cm^{-1} (aryl carbonyl); NMR ($CDCl_3$) δ 1.25 (t, 3 H, J = 7 Hz, $-COOCH_2CH_3$), 1.4–1.9 (m, 8 H, $-COCH_2CH_2CH_2-CH_2CH_2CH_2COOCH_2CH_3$), 2.31 (t, 2 H, J = 7 Hz, $-COCH_2CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 3.05 (t, 2 H, J = 7 Hz, $-COCH_2CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 3.98 (s, 2 H, C_9-H of fluorene), 4.13 (q, 2 H, J = 7 Hz, $-COOCH_2CH_3$), 7.2–8.5 (m, 7 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 350 with the base peak appearing at 193, corresponding to fluorenyl acylium ion.

8-(2-Fluorenyl)-7-oxooctanoic acid ethyl ester (3 g, 8.5 mmol) was dissolvedd in toluene (100 mL) and added to a 500-mL round-bottom flask already containing amalgamated zinc (18 g, 1:6 molar ratio, w/w) in concentrated hydrochloric acid (100 mL). The reaction mixture was refluxed for 9 h when another lot of hydrochloric acid (60 mL) was added. Both the organic and aqueous layers were separated, and the aqueous layer was extracted with ether. Both the toluene and ether layers were mixed, washed with water and then a saturated solution of sodium chloride, and finally dried over anhydrous sodium sulfate. Excess of ether was distilled and toluene was removed by vacuum distillation. The residue so obtained was crystallized from petroleum ether to give pure 8-(2-fluorenyl)octanoic acid ethyl ester (2 g) in 72% yield, mp 45–46 °C; IR 1740 cm^{-1} (ester carbonyl); NMR ($CDCl_3$) δ 1.27 (t, 3 H, J = 7 Hz, $-COOCH_2CH_3$), 1.4–1.9 (m, 10 H, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 2.30 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOCH_2CH_3$), 2.70 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2CH_2-CH_2COOCH_2CH_3$), 3.87 (s, 2 H, C_9-H of fluorene), 4.12 (q, 2 H, J = 7 Hz, $-COOCH_2CH_3$), 7–8 (m, 7 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 336 with the base peak appearing at 179 corresponding to fluorenyl–$CH_2^{\bullet+}$.

8-(2-Fluorenyl)octanoic acid ethyl ester (500 mg, 1.49 mmol) was dissolved in 10% methanolic potassium hydroxide (25 mL), and the reaction mixture was refluxed for 6 h, concentrated, and dumped in water. It was neutralized with hydrochloric acid. The residue was filtered, dried under vacuum, and crystallized from benzene–petroleum ether mixture to give creamish needles of pure (2-fluorenyl)octanoic acid (300 mg) in 70% yield: HPLC R_t 3.2 min; mp 114–115 °C; IR 1715 cm^{-1} (acid carbonyl); NMR ($CDCl_3$) δ 1.34 (m, 6 H, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$), 1.62 (m, 4 H, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$), 2.3 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$), 2.65 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$), 3.85 (s, 2 H, C_9-H of fluorene), 7–8 (m, 7 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 308 with the base peak appearing at 179 corresponding to fluorenyl–$CH_2^{\bullet+}$. Anal. Calcd for $C_{21}H_{24}O_2$: C, 81.81; H, 7.79. Found: C, 81.50; H, 7.71.

4-(7-n-Butylfluoren-2-yl)butyric Acid (V, C4A-FL-C4). Fluorene was acylated with butyric anhydride according to the procedure of Buu-Hoi and Cangihant (1946) to obtain 2-butyroylfluorene in 52% yield, mp 116 °C. The acylated product was then subjected to Wolff–Kishner reduction according to the procedure of Huang (1946) to get 2-*n*-butylfluorene, mp 65–66 °C. Dry 2-*n*-butylfluorene (8 g, 0.036 mol) was dissolved in nitrobenzene (50 mL), and succinic anhydride (3.99 g, 0.04 mol) was added to it. The reaction was stirred well with cooling at 0 °C. Aluminium chloride (9.6 g, 0.072 mol) was added in portions with constant stirring. The mixture was stirred at 0 °C for 1 h and then kept at 4 °C for the next 36 h. The reaction mixture was then dumped in crushed ice and acidified with dilute hydrochloric acid. The nitrobenzene was removed by steam distillation. The product was filtered, dried, and crystallized from acetic acid to give 4-(7-*n*-butylfluoren-2-yl)-3-oxobutyric acid, (10.18 g) in 88% yield: HPLC R_t 5.2 min; mp 182–183 °C; IR 1710 cm^{-1} (acid carbonyl), 1680 cm^{-1} (aryl carbonyl); NMR ($CDCl_3$) δ 0.99 (t, 3 H, J = 7 Hz, $-CH_2CH_2CH_2CH_3$), 1.2–1.9 (m, 4 H, $-CH_2CH_2CH_2CH_3$), 2.6–3.0 (m, 4 H, $-COCH_2CH_2COOH$ and $-CH_2CH_2CH_2CH_3$), 3.4 (t, 2 H, J = 7 Hz, $-COCH_2CH_2COOH$), 3.95 (s, 2 H, C_9–H of fluorene), 7.17–8.1 (m, 6 H, aryl protons). Mass spectrum gave the molecular ion peak at m/z 322 and the base peak at 249 corresponding to the acylium ion.

4-(7-*n*-Butylfluoren-2-yl)-3-oxobutyric acid (5 g, 0.015 mol) was dissolved in toluene (300 mL) and added to a 1-L round-bottom flask, already containing zinc amalgam (30 g, 1:6 molar ratio, w/w) in concentrated hydrochloric acid (150 mL). The reaction mixture was then refluxed for 8 h, after adding another lot of concentrated hydrochloric acid (100 mL). The organic layer was then separated, and the aqueous layer was extracted with chloroform. Both toluene and chloroform layers were mixed and washed with water and then a saturated solution of sodium chloride, followed by drying over anhydrous sodium sulfate. Excess of chloroform was distilled on a water bath, and the toluene was vacuum distilled. The solid residue so obtained was crystallized from benzene–petroleum ether to give 4-(7-*n*-butylfluoren-2-yl)butyric acid (3.9 g) in 81% yield: HPLC R_t 3.7 min; mp 159–160 °C; IR 1700 cm^{-1} (acid carbonyl); NMR ($CDCl_3$) δ 0.95 (t, 3 H, J = 7 Hz, $-CH_2CH_2CH_2CH_3$), 1.38 (p, 2 H, J = 7 Hz, $-CH_2CH_2-CH_2CH_3$), 1.63 (p, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_3$), 1.99 (p, 2 H, J = 7 Hz, $-CH_2CH_2CH_2COOH$), 2.37 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2COOH$), 2.66 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2COOH$), 2.72 (t, 2 H, J = 7 Hz, $-CH_2CH_2CH_2CH_3$), 3.82 (s, 2 H, C_9-H of fluorene), 7.16 (d, 2 H, J = 7.5 Hz, C_4-H and C_5-H of fluorene), 7.33 (s, 2 H, C_1-H and C_8-H of fluorene), 7.64 (d, 2 H, J = 7.5 Hz, C_3-H and C_6-H of fluorene). Mass spectrum gave the molecular ion peak at m/z 308 with the base peak appearing at 179 corresponding to fluorenyl–$CH_2^{\bullet+}$. Anal. Calcd for $C_{21}H_{24}O_2$: C, 81.81; H, 7.79. Found: C, 81.87; H, 7.74.

Fluorescence Studies. All solvents used were of spectroscopy grade from Merck, Bombay. Steady-state fluorescence measurements were carried out on a Shimadzu RF 540 fluorescence spectrometer. All fluorescence intensity measurements were corrected for dilution and light scattering.

Phosphatidylcholine (PC) was isolated from hen egg yolk according to the procedure of Singleton et al. (1965). It was stored as a $CHCl_3$ solution and made free of any oxidized impurity by washing with water and precipitation with acetone

according to the procedure of Poghossian and Nalbandyan (1980). The PC vesicles were prepared by first forming a thin film by using a rotary evaporator and then drying under high vacuum (0.1 mm) for 10 h. A 10 mM NaCl solution was then added and vortexed. This suspension was then sonicated with a Branson B-30 sonicator using a microtip for 5 min at 4 °C. The resulting solution was centrifuged at 10000*g* for 15 min. The PC concentration in the vesicle preparation was 0.5 mM. An aliquot of 2 mM solution of fluorenyl fatty acids in methanol was added to the PC vesicles so that the final phospholipid to fluorenyl fatty acid molar ratio was 200:1 and the final methanol concentration was 0.12% v/v. The vesicle preparation was then incubated at 25 °C for 45 min. Fluorescence spectra were then recorded with excitation wavelength set at 272 nm. Excitation and emission slits were 5 and 10 nm, respectively. The fluorescence spectra of all fluorenyl fatty acids in *n*-hexane and on being incorporated in phosphatidylcholine vesicles were found to be similar.

A 4 M solution of KI containing a small amount of 10 mM $Na_2S_2O_3$ solution added to reduce the formation of I_2 and I_3^- was used for iodide quenching studies. Small aliquots (8 μL) of the KI solutions were added successively to the PC vesicle preparation and the emission spectra recorded. Similarly, in the case of Cu(II) quenching studies, 4-μL aliquots of a 1.25 mM solution of copper sulfate were added successively. 9,10-Dibromostearic acid was prepared by addition of bromine to a CCl_4 solution of oleic acid at 0 °C according to the procedure of Nevenzel and Howton (1957). A TLC single-spot sample of 9,10-dibromostearic acid was obtained by chromatographing the crystalline sample on silica gel. A 5.4 mM solution of 9,10-dibromostearic acid in methanol was used for quenching studies, and small aliquots (4 μL) were added successively to the vesicle preparation. After each addition of 9,10-dibromostearic acid the sample was incubated for 45 min at 25 °C to ensure uptake of the quencher before recording the emission spectrum. Quenching experiments using 12-bromostearic acid were carried under identical conditions.

Fluorescence Polarization Studies. The fluorescence polarization studies were carried out at 0, 25, 33, and 50 mol % of cholesterol. Vesicles from PC and cholesterol were prepared by adding the appropriate mole percent of cholesterol in chloroform to the PC solution in chloroform before drying under vacuum and sonicating in 10 mM NaCl as described above. The sonicating period had to be increased to 8 min to obtain clear solutions in the PC/cholesterol vesicle preparation. The concentration of PC in these vesicle preparations was 0.25 mM, and the PC to fluorenyl fatty acid molar ratio was maintained at 450:1 by adding a 1 mM solution of the fluor in methanol as described above. Fluorescence polarization data were obtained by setting the excitation and emission wavelengths at 272 and 315 nm, respectively. In the fluorescence polarization measurements the intensities of horizontal and vertical components of the emitted light ($I_\parallel$ and $I_\perp$) were corrected for the contribution of scattered light ($I_\parallel'$ and $I_\perp'$) determined independently for a referenced vesicle preparation to which the fluorenyl fatty acid was not added. The grating correction factor G for the polarizer was obtained by setting the excitation polarizer vertical and measuring the fluorescence intensity with the emission polarizer set parallel ($I_\parallel''$) and perpendicular ($I_\perp''$), respectively, where $G = (I_\parallel''/I_\perp'')$. The corrected fluorescence polarization value p is thus obtained by using the equation:

$$p = \frac{(I_\parallel - I_\parallel') - (I_\perp - I_\perp')G}{(I_\parallel - I_\parallel') + (I_\perp - I_\perp')G}$$

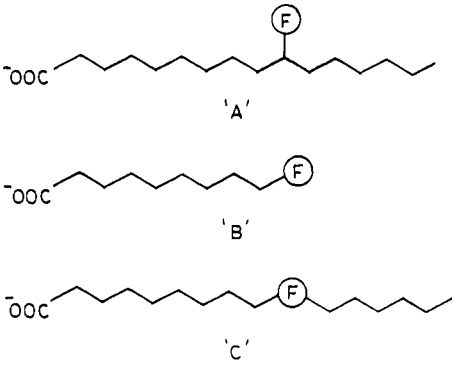

FIGURE 1: A schematic representation of the design of fluorescent fatty acids.

Steady-state fluorescence anisotropy r was calculated from the polarization values by using the equation:

$$r = 2p/(3 - p)$$

The rotational correlation time ϕ was obtained by using the following general form of Perrin's (1926) equation:

$$r_0/r - 1 = \tau/\phi$$

where r_0 is the limiting anisotropy and τ is the fluorescence lifetime. The r_0 was computed from a Perrin plot by measuring the anisotropy of these fluorenyl fatty acids in various glycerol–water mixtures. The τ measurements were carried out on a SP-70 nanosecond fluorescence spectrometer, Applied Photophysics, England, using a single photon counting technique. The samples were excited with an excitation wavelength of 272 nm. Results were deconvoluted as single exponentials by using a nonlinear least-squares fitting program.

RESULTS

Design and Synthesis of Fluorenyl Fatty Acids. Fluorescent fatty acids having variable distance between the carboxyl group and the fluorescent chromophore have been used for studying the membrane hydrophobic core at different depths. Most notable in this class are *n*-(9-anthroyloxy) fatty acids (n = 2, 6, 9, 12) wherein the anthroyloxy group is attached via an ester linkage to various positions on the alkyl chain (Thulborn & Sawyer, 1978). Fluorescence being a highly sensitive and informative technique, these fluorescent probes have been extensively used (Thulborn et al., 1979; Lakowicz, 1981; Thulborn, 1981; Vincent et al., 1982; Vincent & Gallay, 1984; Kleinfeld & Lukacowicz, 1985; Storch & Kleinfeld, 1986). But like any other membrane hydrophobic core reporter molecule, e.g., ESR probes such as doxylstearic acids (Marsh & Smith, 1973) and fatty acids incorporating photoactivatable reagents (Gaffney, 1985), they have to be critically evaluated for the possible membrane perturbation caused by them. To achieve minimal perturbation, we have looked into the molecular design of these fatty acids. The schematic representation given in Figure 1 provides a simple model for classifying existing depth-dependent probes and synthesizing new ones. Thus, the anthroyloxy fatty acids can be classified in category A and the pyrenyl (Waka et al., 1980; Jones & Lentz, 1986) and anthracene (de Bony & Tocanne, 1983; Vincent et al., 1985; Molotkovsky, 1984) fatty acids (Figure 2) in category B. As mentioned earlier, the probes belonging to category B have come up more recently and appear to be less perturbing compared to the pendent group probes belonging to category A. At this stage it was argued that attachment of a hydrophobic tail to the probes belonging to category B would help in better alignment of these probes in membranes and permit a more optimal depth-dependent assessment of

Table I: Spectral Characteristics of Fluorenyl Fatty Acids

probe	absorbance (ϵ)[a]			emission λ_{max} (nm)[b]		polarization p		PC vesicles	
				methanol	PC vesicles	PC vesicles	PC/cholesterol vesicles (2:1)	τ (10^{-9} s)	ϕ (10^{-9} s)
C2A-FL (I)	266 (27 500)	292 (9150)	303 (11 800)	311	313				
C4A-FL (II)	266 (25 200)	293 (7875)	305 (10 395)	313	314	0.064	0.073	17.70	3.74
C6A-FL (III)	265 (26 750)	293 (8000)	304 (10 500)	315	315	0.084	0.10	15.58	4.66
C8A-FL (IV)	265 (22 450)	293 (6900)	304 (10 000)	315	314	0.09	0.099	18.76	6.15
C4A-FL-C4 (V)	272 (27 000)	297 (8250)	308 (10 450)	320	319	0.146	0.16	18.55	13.23

[a] Absorbance values in nm recorded in methanol. The molar extinction coefficients (M^{-1} cm^{-1}) are given in parentheses below the individual wavelengths. [b] Excitation wavelength is 272 nm.

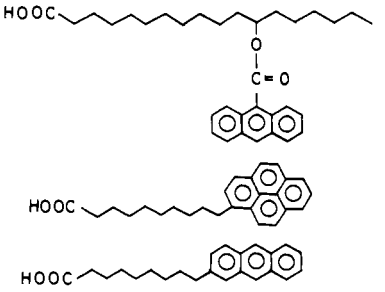

FIGURE 2: Structures of 12-(9-anthroyloxy)stearic acid, 10-(1-pyrenyl)decanoic acid, and 9-(2-anthryl)nonanoic acid.

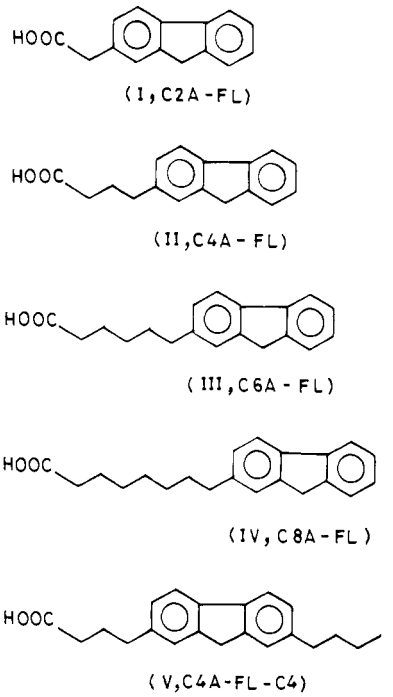

FIGURE 3: Structure of fluorenyl fatty acids prepared.

membranes. Category C represents such probes. To test this hypothesis, we have synthesized four fluorenyl fatty acids (Figure 3, I–IV) belonging to category B and one fluorenyl fatty acid (Figure 3, V) belonging to category C.

The fluorenyl fatty acids (I–IV, Figure 3) reported here were prepared by Friedel–Craft's acylation of fluorene followed by Wolff–Kishner or Clemmensen reduction of the intermediate keto ester (Figure 4). The fluoren-2-ylbutyric acid with a hydrophobic *n*-butyl tail at C-7, i.e., compound V in Figure 3, was prepared with 2-*n*-butylfluorene as starting material (Figure 4). All these compounds were purified to homogeneity as indicated by TLC and HPLC analysis. Further, the spectral

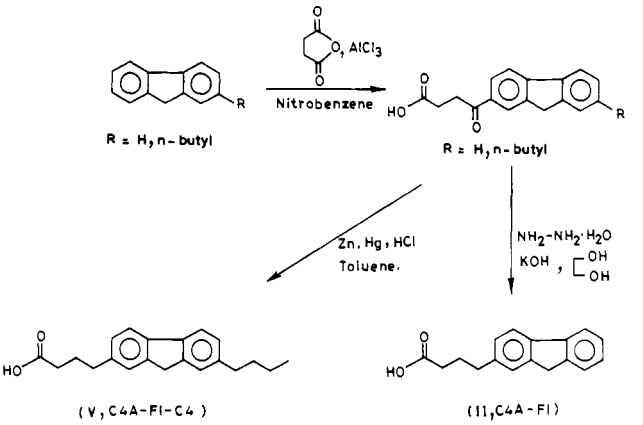

FIGURE 4: Scheme for synthesis of (2-fluorenyl)butyric acid (II, C4A-FL) and (7-*n*-butylfluoren-2-yl)butyric acid (V, C4A-FL-C4).

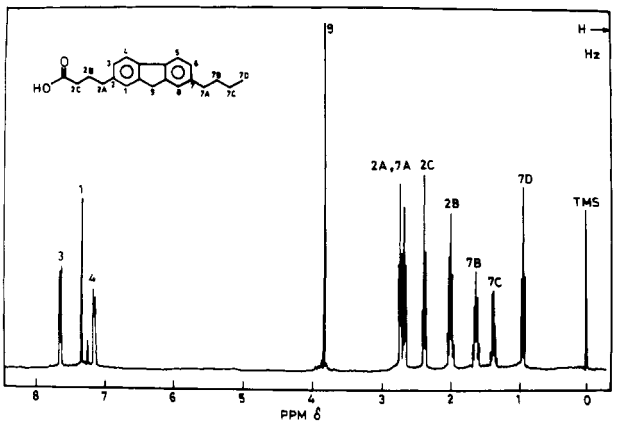

FIGURE 5: Proton NMR spectrum of (7-*n*-butylfluoren-2-yl)butyric acid (V, C4A-FL-C4).

data fully corroborated the structure of the fluorenyl fatty acids reported here. The NMR spectrum of C4A-FL-C4 (V) is given in Figure 5. It is interesting that all the proton signals for this fatty acid can be assigned and thus this compound could also prove to be useful as a NMR probe.

UV Absorption and Emission Spectra. The absorption spectra of fluorene, C4A-FL (II), and C4A-FL-C4 (V) are given in Figure 6. The absorption bands beyond 250 nm undergo a red shift on substitution of fluorene at C-2 though their extinction coefficients are similar (Table I). In the case of C4A-FL-C4 (V) additional substitution at C-7 leads to a still further shift, though the overall shape of the spectrum is similar to that of fluorene. The excitation and emission bands for fluorene in methanol appear at 272 and 315 nm, respectively (Horrocks & Brown, 1970). All the fluorenyl fatty

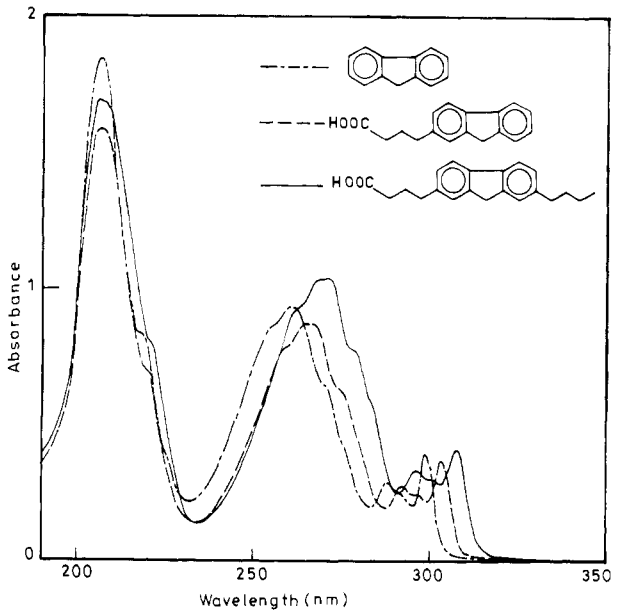

FIGURE 6: UV absorption spectra of 20 μM fluorene (-·-), C4A-FL (---), and C4A-FL-C4 (—) in methanol.

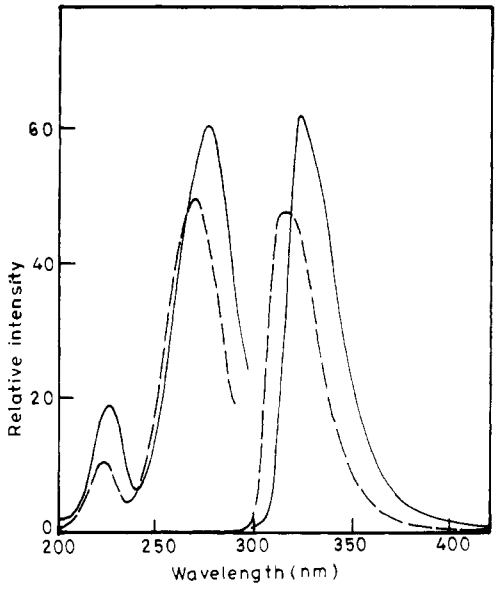

FIGURE 7: Excitation and emission spectra of C4A-FL (---) and C4A-FL-C4 (—) in phosphatidylcholine (PC) vesicles. The PC concentration in the vesicle was 0.5 mM. An aliquot (3 μL) of a 2 mM solution of the probe in methanol was added to the PC vesicle preparation and the solution incubated for 45 min at 25 °C. The PC:probe molar ratio was 250:1 and methanol concentration 0.1% (v/v). The excitation spectrum was obtained by setting the emission wavelength at 315 nm, and the emission spectrum was obtained by setting the excitation wavelength at 272 nm.

acids reported here (I–V) on excitation at 272 nm gave rise to emission maximas between 310 and 320 nm in methanol (Table I). The emission spectra of these compounds were also recorded in other solvents of varying polarity like hexane, chloroform, and water. The solubility of these fluorenly fatty acids being very poor in water made it difficult to record spectra in this solvent. The increase in polarity led to practically no change in the emission maxima. These fatty acids could also be conveniently partitioned into PC vesicles wherein similar excitation and emission spectra were observed (Figure 7).

Fluorescence Quenching Studies. The orientation of the fluorescent probes in membranes has been best studied by following the depth-dependent quenching of their fluorescence

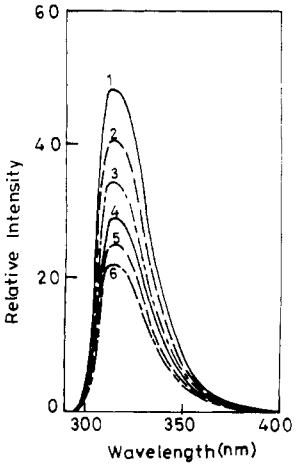

FIGURE 8: Effect of increasing KI concentration on the fluorescence intensity of C4A-FL bound to PC vesicles. The probe to lipid molar ratio was 1:200. The iodide concentrations (mM) from top to bottom are 1 (0), 2 (10.66), 3 (21.32), 4 (32.0), 5 (42.66), and 6 (53.3 mM). The excitation wavelength is 272 nm.

in membranes (Blatt & Sawyer, 1985). The orientation of *n*-(9-anthroyloxy) fatty acids (n = 2, 6, 9, 12) in artificial membranes has been studied by using this technique (Thulborn, 1981; Haigh et al., 1979). To get an insight into the orientation of fluorenyl fatty acids in PC vesicles, we have studied their fluorescence quenching in vesicles using extrinsic water-soluble quenchers like iodide and Cu(II) and intrinsic lipid-soluble quenchers like 12-bromostearic acid and 9,10-dibromostearic acid. The quenching of C4A-FL in PC vesicles by iodide is given in Figure 8, which indicates a clear concentration-dependent quenching. The association of a fluorophore F with a quencher Q can be described by the following relationship between the intensities of fluorescence in the absence (I_0) and presence (I) of the quenching agent:

$$(I_0/I) - 1 = K_{SV}[Q]$$

where [Q] is the quencher concentration and K_{SV} is the corresponding equilibrium constant, often referred to as Stern–Volmer's constant (Stern & Volmer, 1919). A plot of $(I_0/I) - 1$ vs quencher concentration gives a linear plot passing through the origin. This equation is valid only for dyanamic quenching arising from molecular collision. In case a static component is involved in quenching, the Stern–Volmer plot deviates from linearity (Blatt & Sawyer, 1985).

We have studied the quenching of fluorenyl fatty acid fluorescence in PC vesicles using water-soluble quenchers like iodide and copper. These quenchers are highly soluble in aqueous environments relative to the apolar environment of the bilayer. Consequently, very few ions penetrate beneath the surface of the bilayer and high concentrations of the quencher are required to quench fluorescent chromophores embedded in the bilayer. Taking advantage of this low relative solubility in bilayers, both iodide (Cranney et al., 1983) and Cu(II) (Thulborn & Sawyer, 1978) have been used to assess the transverse location of fluorescent probes in the membrane. The Stern–Volmer plots for iodide quenching of the fluorescence of the four fluorenyl fatty acids (I–IV) in PC vesicles are given in Figure 9. Sodium iodide, being highly water soluble, barely partitions into the membrane and thus quenches the shallowest probe C2A-FL flourescence maximally whereas the deepest probe C8A-FL is minimally quenched. The quenching order C2A-FL > C4A-FL > C6A-FL > C8A-FL suggests that these probes are oriented as expected in the membrane. The Cu(II) quenching data also supported this conclusion. Interestingly, C4A-FL-C4 appears to be probing

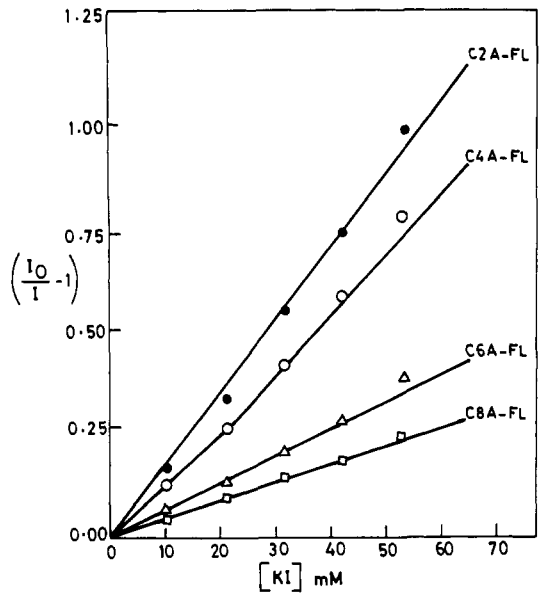

FIGURE 9: Stern–Volmer plots for quenching of fluorenyl fatty acids C2A-FL (●), C4A-FL (○), C6A-FL (△), and C8A-FL (□) bound to PC vesicles by KI. I_0 is the fluorescence intensity in the absence of the quencher and I the intensity in the presence of the quencher.

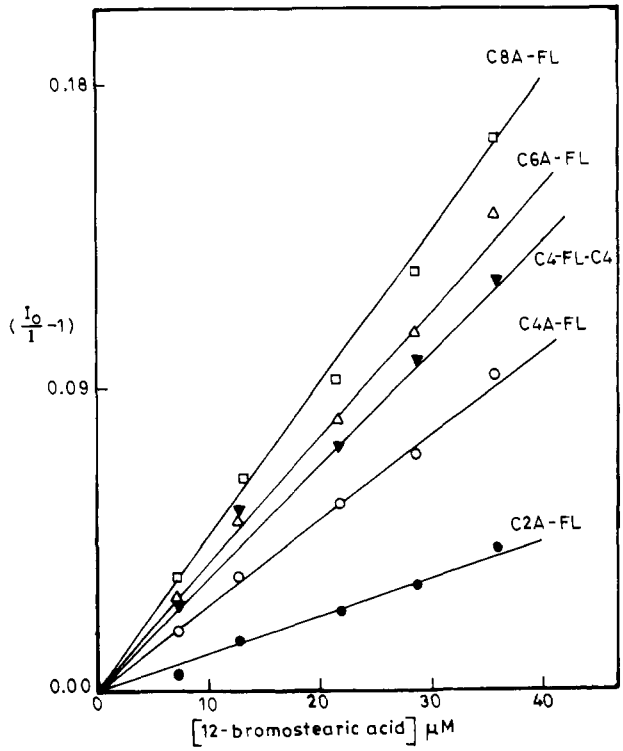

FIGURE 10: Stern–Volmer plots of 12-bromostearic acid quenching of the fluorenyl fatty acids C2A-FL (●), C4A-FL (○), C4A-FL-C4 (▼), C6A-FL (△), and C8A-FL (□) bound to PC vesicles.

deeper than C4A-FL, suggesting a better alignment of this probe in membranes.

Brominated fatty acids and phospholipids have been recently used to study depth-dependent quenching in a number of systems (East & Lee, 1982; Ludi et al., 1985; Jain & Maliwal, 1985, Everett et al., 1986). The intrinsic quenchers 9,10-dibromostearic acid and 12-bromostearic acid were used as these quenchers would readily partition into the membrane and quench the fluorenyl fatty acid fluorescence in a reverse order relative to iodide and Cu(II), the C8A-FL now being most proximal to the quencher. Figure 10 gives the Stern–Volmer plot for 12-bromostearic acid quenching, clearly indicating the reversal of quenching order, i.e., C8A-FL > C6A-FL > C4A-FL > C2A-FL. Similar data were obtained with 9,10-dibromostearic acid. A plot of the Stern–Volmer constants (K_{SV}) derived from these plots was plotted against the length of the carbon chain attached to the fluorenyl fatty acids and the position of C4A-FL-C4 marked by an arrow (Figure 11). It is clear from these data that the fluorenyl fatty acids (I–IV) are correctly oriented in the membrane, but C4A-FL-C4 probes deeper than C4A-FL.

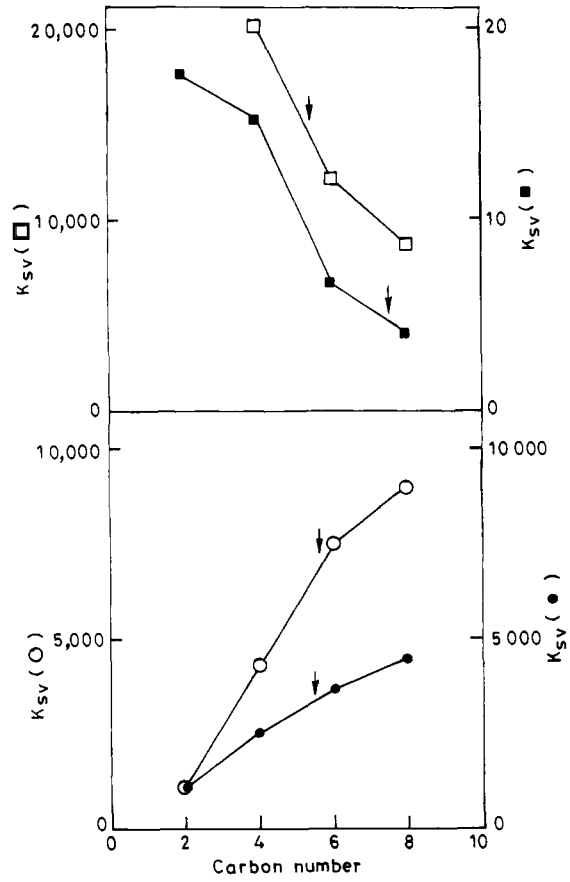

FIGURE 11: Plots of the Stern–Volmer quenching constants (K_{SV}) obtained for the quenching of fluorenyl fatty acids in PC vesicles preparations by KI (■), Cu(II) (□), 9,10-dibromostearic acid (○), and 12-bromostearic acid (●) as quenchers versus the length of the alkyl acid chain attached to fluorene. The position of C4A-FL-C4 is indicated by the arrow.

Polarization Studies. The fluorescence polarization of these probes was also determined (Table I) in PC vesicles in order to get an estimate of their degree of immobilization in membranes. As expected, with an increase in the chain length while going from C4A-FL to C8A-FL one observes an increase in polarization. Nevertheless, it is interesting to note that C4A-FL-C4 has higher polarization value when compared with C8A-FL of similar length. The fluorescence life time τ of the fluorenyl fatty acids in PC vesicles was also measured and was found to be similar (Table I). The fluorescence lifetime data permitted evaluation of the rotational correlation time ϕ for these probes (Table I). Again, one sees from these data that while the ϕ for C8A-FL > C6A-FL > C4A-FL as expected on the basis of increased length of the compounds, the higher rotational correlation time value for C4A-FL-C4 relative to C8A-FL indicates much better immobilization of the former in PC vesicles.

Cholesterol is known to regulate fluidity of membranes. This variation in fluidity resulting from an increase in cholesterol concentration in membrane preparations like PC vesicles can be monitored by the change in polarization of

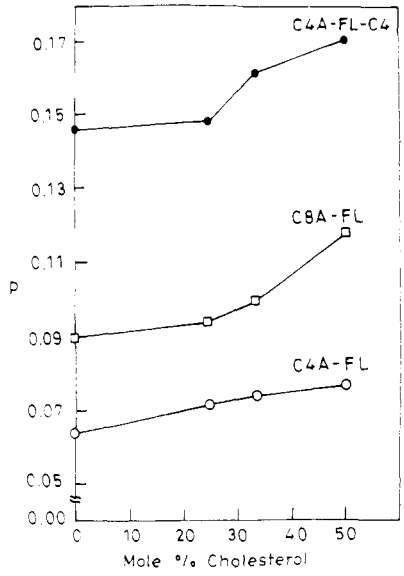

FIGURE 12: Plots of fluorescence polarization values of the fluorenyl fatty acids C4A-FL (○), C8A-FL (□), and C4A-FL-C4 (●) incorporated in PC and PC/cholesterol vesicles versus mole percent cholesterol.

embedded fluorescent probes like DPH (Shinitzky & Inbar, 1976). A similar increase in polarization with these fluorenyl fatty acids was also observed with increasing concentration of cholesterol in PC vesicles (Figure 12). It is interesting to note that, even at variable concentrations of cholesterol, C4A-FL-C4 has a much higher value of polarization compared to C4A-FL and C8A-FL.

Discussion

The fluorenyl fatty acids (I–V) reported here for depth-dependent analysis of membranes do not contain any polar linkage site as in the case of *n*-(9-anthroyloxy) fatty acids (Thulborn & Sawyer, 1978; Thulborn, 1981). Consequently, the probes reported here are likely to minimize the membrane perturbation. The fluorescence quenching data indicate that the fluorenyl fatty acids (I–IV) probe the membrane at different depths as expected from their transverse location. Thus C2A-FL is closer to the lipid–water interface and C8A-FL the farthest. Further, based on new design criterion, it has been possible to make probes of category C (Figure 1) and study their orientation in the membrane. Thus on the basis of transverse location of the fluorene chromophore in the fluorenyl fatty acids (I–V), one would have expected C4A-FL and C4A-FL-C4 to probe the membrane at similar depths, but the fluorescence quenching studies with four different quenchers indicated that the fluorenyl group in C4A-FL-C4 is situated deeper in the membrane relative to its disposition in C4A-FL. On the other hand, on the basis of length alone, one would expect C4A-FL-C4 to be more strongly immobilized in the membrane relative to C4A-FL. The polarization and the rotational correlation time data substantiate this statement, but the fact that C4A-FL-C4 has these values higher than even C6A-FL and C8A-FL (Table I) indicates that the addition of an *n*-butyl chain to C4A-FL certainly assists in properly orienting and immobilizing this depth-dependent probe. In brief, probes belonging to category C (Figure 1) would be more effective in probing membranes at different depths. Thus, in principle a C2 or C4 chain can be attached to C6A-FL or a pyrene- or anthracene-based fatty acids (Figure 2) to position the fluorescent chromophore for optimal depth-dependent probing in membranes. The synthesis and use of fluorescent probes belonging to category C (Figure 1) will thus provide more useful information, especially when compared to probes belonging to category A, e.g., anthroyloxy fatty acids (Figure 2), wherein recent NMR data indicate that the fluorophore in these fluorescent probes on incorporation in membranes has a slower and a faster motion component, and it is only the latter component that is detected by fluorescence depolarization. Further, the normal membrane lipids move on a time scale that corresponds to the slower component mentioned above, which makes the study of membrane dynamics debatable using probes belonging to category A (Kuroda et al., 1986).

We must add here that the fluorenyl fatty acid probes (I–V) reported here would be more useful for studies in artificial membranes and would have only limited utility in natural membranes, as the intrinsic fluorescence of proteins emanating from tyrosine and tryptophan residues strongly overlaps with the fluorene emission spectrum. It may be possible under favorable circumstances to selectively excite fluorenyl chromophore at 272 nm or at lower wavelengths without exciting the tryptophan residues, which absorb around 285 nm, but then such application would be more selective on the basis of the experimental system. We were aware of this general limitation of fluorene as a fluorescent probe, but we had different reasons for pursuing fluorene-based fatty acids as described below. Nevertheless, this limitation in no way affects the basic molecular design criterion developed and demonstrated with the fluorenyl fatty acids.

Our interest in fluorenyl fatty acids stems from the fact that we have recently reported diazofluorene (DAF) as a new photoactivatable reagent for labeling membrane hydrophobic core in both artificial (Anjaneyulu & Lala, 1982; Anjaneyulu et al., 1984) and natural (Pradhan & Lala, 1987) membranes. These studies indicated that DAF effectively labels membrane-spanning domains of integral membrane proteins in human erythrocytes (Pradhan & Lala, 1987). To further exploit the potential of DAF as a photoactivatable reagent, we wanted to synthesize phospholipids incorporating DAF-based fatty acids. These phospholipids could be effectively used for studying reconstituted membrane-bound proteins as in the case of phospholipids derived from fatty acids containing an aryl azide (Bisson et al., 1979), phenyldiazirine (Takagaki et al., 1983), or [(trifluoromethyl)phenyl]diazirine (Brunner et al., 1983) group. In most of these cases the photoactivatable group is attached as a terminal group at the ω-carbon atom of the fatty acid via an ether or amide linkage. The preference for this design is possibly a result of easy availability of ω-hydroxy and ω-amino fatty acids which provide convenient linkage sites for photoactivable reagents. The phospholipids prepared from these fatty acids lead to the introduction of a polar group in the membrane preparation, and the photoactivatable groups in some cases have been reported to loop back to the interface and label the amino acids of the integral membrane proteins, e.g., glycophorin (Ross et al., 1982), around the interfacial region. This is not surprising in view of the fact that a polar group will try to move out of the hydrophobic core of the membrane. This problem has been recently addressed in a paper by Harter et al. (1988) wherein they report a [(trifluoromethyl)phenyl]diazirine-based phospholipid, which does not contain an ether link between the photoactivatable group and the fatty acyl chain. Despite these molecular design problems the photoactivatable phospholipids have proved to be very useful in probing the topography of membrane-bound proteins. In order to get over the problems mentioned above, we decided to first design fluorenyl fatty acids and test their orientation in membranes before converting

them to the diazofluorene analogues. The availability of fluorenyl fatty acids with a hydrophobic tail, i.e., category C in Figure 1, thus not only provides a fluorescent probe but also a suitable precursor for diazofluorene-based photoactivatable reagents. The synthesis of these reagents is currently in progress in our laboratory.

ACKNOWLEDGMENTS

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Registry No. I, 36950-95-5; II, 7250-03-5; III, 116971-36-9; IV, 116971-37-0; V, 116996-80-6; ethyloxalyl chloride, 4755-77-5; ethyl (2-fluorenyl)oxoacetate, 37414-53-2; fluorene, 86-73-7; succinic anhydride, 108-30-5; 4-(2-fluorenyl)-3-oxobutyric acid, 116971-38-1; ethyladipoyl chloride, 1071-71-2; 6-(2-fluorenyl)-5-oxohexanoic acid ethyl ester, 116971-39-2; 6-(2-fluorenyl)hexanoic acid ethyl ester, 116971-40-5; ethylsuberoyl chloride, 14113-02-1; 8-(2-fluorenyl)-7-oxooctanoic acid ethyl ester, 116971-41-6; 8-(2-fluorenyl)octanoic acid ethyl ester, 116971-42-7; butyric anhydride, 106-31-0; 2-butyroylfluorene, 60505-47-7; 2-*n*-butylfluorene, 42946-58-7; 4-(7-*n*-butylfluoren-2-yl)-3-oxobutyric acid, 116971-43-8; potassium iodide, 7681-11-0; copper, 7440-50-8; 9,10-dibromostearic acid, 19117-94-3; 12-bromostearic acid, 116971-44-9.

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