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Effect of Cholesterol on the Ethanol-Induced Interdigitated Gel Phase in Phosphatidylcholine: Use of Fluorophore Pyrene-Labeled Phosphatidylcholine†

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ABSTRACT: It is now recognized that many amphiphilic molecules such as ethanol can induce the formation of the fully interdigitated gel phase ($L_\beta I$) in phosphatidylcholines (PC's). In the present study, we have developed a simple detection method for the $L_\beta I$ phase using pyrene-labeled PC (PyrPC), which is a PC analogue with a covalently coupled pyrene moiety at the end of one of its acyl chains. The intensity ratio of its fluorescence vibrational bands is a reflection of the polarity of the environment of the fluorophore. We have tested this fluorophore in several established interdigitated lipid systems, including 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (1,2-DPPC) in the presence of high concentrations of ethanol and 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (DHPC) and 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine (1,3-DPPC) in the absence of any additives. We have found in each of these systems that the ratio of the intensities of band III (387.5 nm) to band I (376.5 nm) is sensitive to the lipid phase change from the noninterdigitated L_β' phase to the interdigitated $L_\beta I$ phase. By comparison of the III/I ratios for PyrPC in the lipid systems with the III/I ratios for methylpyrene in organic solvents, it was shown that the polarity of the PyrPC environment in the $L_\beta I$ phase is similar to that of pentanol or ethanol. Using this method, we investigated the effect of cholesterol on the ethanol induction of the interdigitated gel phase in 1,2-DPPC. We found that the ethanol induction of the interdigitated gel phase is prevented by the presence of 20 mol % cholesterol.

It is now well established that saturated symmetrical and asymmetrical phosphatidylcholines (PC's)[1] can exist in the interdigitated gel phase ($L_\beta I$) under a variety of conditions (McDaniel et al., 1983; Serrallach et al., 1983; Huang et al., 1983; McIntosh et al., 1983, 1984; Simon & McIntosh, 1984; Hui et al., 1984; Ruocco et al., 1985; Cunningham & Lis, 1986; Hui & Huang, 1986; Mattai et al., 1987; Slater & Huang, 1988). The saturated symmetrical PC's and phosphatidylglycerols (PG's) become interdigitated in the presence of various amphiphilic molecules such as glycerol, methanol, ethylene glycol, benzyl alcohol, chlorpromazine, tetracaine (McDaniel et al., 1983; McIntosh et al., 1983), ethanol (Simon & McIntosh, 1984), and thiocyanate ion (Cunningham & Lis, 1986). Polymyxin B and myelin basic protein can also induce the formation of $L_\beta I$ in PG's (Boggs et al., 1981; Ranck & Tocanne, 1982; Boggs & Rangaraj, 1985). $L_\beta I$ has also been observed in the absence of any inducer in 1,2-dihexadecylphosphatidylcholine (DHPC) (Ruocco et al., 1985; Laggner et al., 1987; Kim et al., 1987), in 1,3-dipalmitoyl-*sn*-glycero-

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[1] Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; 1,2-DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 1,3-DPPC, 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine; 1,2-DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; PyrPC, 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine; MePyr, 1-methylpyrene; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; L_c, crystalline subgel bilayer phase; L_β', tilted-chain bilayer gel phase; P_β', rippled gel phase; L_α, liquid-crystalline bilayer phase; $L_\beta I$, interdigitated gel phase; DSC, differential scanning calorimetry.

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2-phosphocholine (1,3-DPPC) (Serrallach et al., 1983), and in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (1,2-DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (1,2-DSPC) under hydrostatic pressure (Braganza & Worcester, 1986).

Interdigitation can be directly detected by X-ray diffraction (Ranck & Tocanne, 1982; Serrallach et al., 1983; McDaniel et al., 1983; McIntosh et al., 1983, 1984; Simon & McIntosh, 1984; Hui et al., 1984; Ruocco et al., 1985; Cunningham & Lis, 1986; Mattai et al., 1987) or neutron diffraction (Braganza & Worcester, 1986). Infrared and Raman spectroscopy (O'Leary & Levin, 1984), electron spin resonance, differential scanning calorimetry (DSC) (Boggs et al., 1989; Boggs & Rangaraj, 1985; Chong & Choate, 1989; Kao et al., 1990; Rowe & Cutrera, 1990), turbidity (Rowe, 1983), and fluorescence spectroscopy (Nambi et al., 1988; Kao et al., 1990) have been used to detect indirectly and/or to study the interdigitated gel phase. However, additional direct, simple methods are needed to make systematic investigations of the conditions and characteristics of interdigitation.

We have been investigating the use of fluorescent probes to detect and study interdigitation. Recently, we reported the first application of a fluorescence method using 1,6-diphenyl-1,3,5-hexatriene (DPH) for the detection of the phase transition from the L_{β}' to the $L_{\beta}I$ phase (Nambi et al., 1988). Pyrene is also one of the fluorescent probes that have provided insight into the structure of membranes. The fluorescence vibrational structure of pyrene is known to be sensitive to medium polarity (Nakajima, 1971). By use of a fluorescent phospholipid having a pyrene group at the end of its acyl chain, it can be expected that the intensities of the fluorescence vibrational bands may be sensitive to the phase change from the noninterdigitated to the interdigitated gel. In the noninterdigitated membrane the pyrene moiety is near the center of the bilayer, whereas in the interdigitated gel the pyrene group is expected to reside in the more polar environment near the interfacial region.

Our initial studies on the ethanol-induced interdigitation of PC's have mainly focused on single-component model membranes and demonstrated that the transition from the noninterdigitated to the interdigitated gel phase is dependent upon the alcohol concentration, the alcohol chain length, the lipid chain length, and temperature (Rowe, 1983, 1985; Rowe et al., 1987; Veiro et al., 1987, 1988; Nambi et al., 1988). However, in order to elucidate the biological implications of the interdigitated structure of membranes, studies should be extended to include other membrane constituents. A particularly important membrane lipid is cholesterol, which is found in mammalian membranes in large proportions.

In the present report, we have studied the intensities of the fluorescent vibrational bands of pyrene-labeled PC in several model membrane systems, which have already been established to be interdigitated by other techniques, in order to evalute the diagnostic capability of this technique. These interdigitated systems include 1,2-DPPC membranes in the presence of high concentrations of ethanol and DHPC and 1,3-DPPC membranes in the absence of any additives. Using this technique, we have then studied the effects of cholesterol on the formation of $L_{\beta}I$ induced by high concentrations of ethanol in 1,2-DPPC.

Materials and Methods

Materials. 1,2-DPPC and cholesterol were purchased from Sigma Chemical Co., St. Louis, MO, and 1,3-DPPC was from Fluka Chemical Corp., Ronkonkoma, NY. DHPC was obtained from Serdary Research Laboratories, Ontario, Canada. 1-Methylpyrene (MePyr) and the fluorescent lipid 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine (PyrPC),

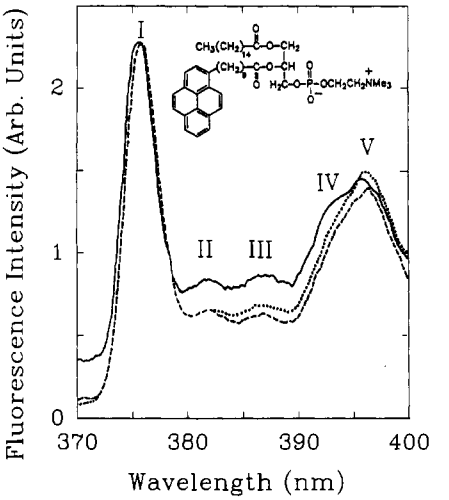

FIGURE 1: Structure of fluorophore PyrPC and fluorescence emission spectra of MePyr in *n*-heptane (solid line) and methanol (dashed line) and of PyrPC in methanol (dotted line) at 20 °C. All curves were normalized to band I. The concentrations of MePyr and PyrPC were 1.2 μM. The excitation wavelength was 325.0 nm (excitation and emission slits of 2 nm).

whose structure is shown in Figure 1, were obtained from Molecular Probes, Eugene, OR. Ethanol (200 proof) was purchased from Publicker Industries, Linfield, PA. Methanol, 1-butanol, 1-pentanol, *n*-heptane, and chloroform of spectroquality grade were obtained from Fisher Scientific, Fair Lawn, NJ. Doubly distilled water was used to make all samples.

Samples. The stock solutions of lipids and fluorophores were prepared in chloroform. The chloroform stock solutions were kept in the freezer under a nitrogen gas atmosphere in the dark until ready to use. The concentration of PyrPC was calculated from the molar extinction coefficient at 342 nm in ethanol of 42 000 $M^{-1} \cdot cm^{-1}$ (Somerharju et al., 1985), and the value of the molar extinction coefficient of MePyr was assumed to be the same as that of PyrPC. Phosphorus concentrations were determined according to Bartlett (1957). The samples were hand-shaken multilamellar vesicles prepared essentially by the method of Bangham et al. (1967). Probe/lipid mixtures dissolved in chloroform were evaporated to dryness under a stream of nitrogen gas and subsequently evacuated for several hours. Buffer solution, composed of 1 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM disodium ethnylenediaminetetraacetate (EDTA), and 0.15 M NaCl (pH 7.2), was added to the thin film containing the lipid and the fluorophore. Nitrogen gas was bubbled to remove any dissolved oxygen, and the lipid was hydrated at about 50 °C. During this incubation, the sample was vortexed periodically. The total lipid and probe concentrations in all the experiments were 0.13 mM and 1.2 μM, respectively (about 1:100 mole ratio). The sample solution, 3.0 mL, was placed in a fluorescence cuvette, and the cuvette was then sealed with a silicon cap. The sample was deaerated by flushing nitrogen gas through the sample solution for about 15 min. Prior to measurements, the samples were incubated at about 50 °C for 30 min in order to avoid the crystalline subgel bilayer phase (L_c) of 1,2-DPPC (Ruocco & Shipley, 1982). The sample was then cooled to about 2 °C prior to the addition of ethanol. Addition of ethanol to the sample was carried out by injecting nitrogen-saturated ethanol into the sample solution through the silicon cap, sealing the cuvette with a microsyringe.

Absorption and Fluorescence Spectroscopy. Absorption spectra were recorded on a Varian/Cary 219 spectrophotometer. All fluorescence experiments were performed on an SLM

8225 digital single-photon-counting spectrofluorometer equipped with a thermostated cuvette holder and connected to an Apple IIe computer. Spectra were recorded on a Hewlett-Packard 7470A plotter. All emission spectra were uncorrected. The temperature was controlled with a programmable Neslab RTE-5DD circulating bath. The temperature of the samples was monitored with an Instrulab Model 700 digital thermometer, with the thermistor placed in a parallel reference cuvette. The sample was magnetically stirred during the measurements. The excitation wavelength was 325.0 nm, and the emission spectrum was scanned in increments of 0.25 nm. The spectral resolution was 2 nm. A control sample prepared without a fluorophore gave less than a 1% scattering signal. Fluorescence intensities were obtained from the strongest intensity on the spectra in the region within ±0.5 nm of the required wavelength.

RESULTS

Fluorescence Spectra of PyrPC in Organic Solvents. Although the peak wavelengths are slightly different (Lianos & Georghiou, 1979), 1-position-substituted pyrenes show vibrational structures similar to the parent pyrene in their monomer fluorescence spectra in solution. The fluorescence emission spectra of MePyr in methanol and *n*-heptane at 25 °C are shown in Figure 1. For the sake of convenience, the five predominant bands are numbered I–V from the shortest wavelength band as established for pyrene (Kalyanasundram & Thomas, 1977). The band numbers I, II, III, IV, and V correspond to the bands having a peak at 376.5, 382.5, 387.5, 393.0 (shoulder), and 397.0 nm, respectively. The spectra in Figure 1 are normalized at the maximum intensity of band I.

In Figure 1, band III shows significant enhancement in intensity and a slight blue shift in the apolar *n*-heptane compared with the more polar methanol. The intensity III/I ratio is used to quantitate the degree of polarity. By comparing the III/I ratios for PyrPC in 1,2-DPPC with the III/I ratios in solvents of known polarity, it is possible to estimate the polarity of the environment around the pyrene moiety in 1,2-DPPC or other membranes. Since PyrPC is insoluble in *n*-heptane, we used the III/I ratios of MePyr in various solvents to provide the standard because the absorption spectra (not shown) are the same and the fluorescence emission spectra for PyrPC and MePyr are similar in methanol, as shown in Figure 1.

Fluorescence Intensity III/I Ratios of PyrPC in Model Membranes. The fluorescence intensity III/I ratios are used to quantitate the polarity of the environment around the PyrPC fluorophore in the lipids. Figure 2 shows the III/I ratios for PyrPC in 1,2-DPPC in the presence and absence of ethanol as a function of temperature. The III/I ratios of MePyr in methanol, ethanol, 1-pentanol, and *n*-heptane as a function of temperature are shown as lines in Figure 2 for reference. They show that in organic solvents the values of the III/I ratio increase linearly with temperature.

The III/I ratios for 1,2-DPPC in the absence of ethanol show a linear increment with increasing temperature, nearly parallel to the lines for mePyr in the organic solvents. There is very little change in polarity at the transition temperatures for the tilted-chain bilayer gel phase ($L_{\beta'}$) to the rippled gel phase ($P_{\beta'}$) pretransition (at 35.0 °C) and the main melting transition from $P_{\beta'}$ to the liquid-crystalline phase (L_α) (at 41.5 °C). The polarity around the pyrene group is between that of 1-pentanol and *n*-heptane. This is an expected result because the 1,2-DPPC membrane is a bilayer throughout these phase changes, and the pyrene group is expected to remain buried within the apolar region of the bilayer. The III/I ratio

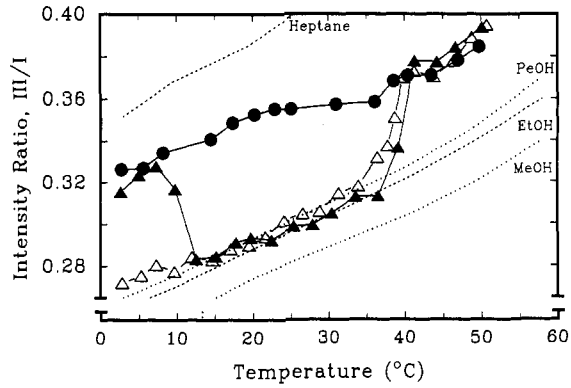

FIGURE 2: III/I ratios for PyrPC in 1,2-DPPC liposomes (symbols and lines) and for MePyr in organic solvents (lines) as a function of temperature: (●) heating sequence without ethanol; (▲) heating sequence with 2.0 M ethanol; (△) cooling sequence performed after heating sequence with 2.0 M ethanol. Lines show the III/I ratio values in organic solvents at 25 °C: MeOH, methanol, EtOH, ethanol; PeOH, 1-pentanol; Heptane, *n*-heptane.

of the pyrene moiety is not affected significantly by the change in acyl-chain mobility or packing; it is only affected by the polarity of its environment.

In the presence of 2.0 M ethanol, the results for PyrPC in 1,2-DPPC are quite different, as shown in Figure 2. The III/I ratios decreased abruptly at about 12 °C, and at about 40 °C they returned to the level of polarity obtained in the absence of ethanol. In the region of about 12–40 °C, the III/I ratios correspond to that of MePyr in ethanol or 1-pentanol, showing that the environment around the pyrene group is much more polar in this temperature region than in the absence of ethanol. The changes in the polarity to and from the more polar environment correspond well with the established temperatures of the 1,2-DPPC transitions from $L_{\beta'}$ to L_βI (at 14 °C) and from L_βI to L_α (at 40 °C) in the presence of 2.0 M ethanol, detected by X-ray diffraction and fluorescence intensity changes of DPH (Nambi et al., 1988; Rowe & Nelson, 1990).

The III/I ratios obtained during cooling performed immediately after the heating run in the presence of ethanol are also depicted in Figure 2. Below about 35 °C, the environment around the pyrene moiety remains polar and does not return to the less polar environment below 12°, indicating that the 1,2-DPPC membrane remains interdigitated in this region. It has previously been shown that, in the presence of ethanol, upon cooling 1,2-DPPC goes from L_α to L_βI in the main transition, but the transition from L_βI to $L_{\beta'}$ is very slow and can not be observed because of its slow kinetics (Nambi et al., 1988). The data shown in Figure 2 for the III/I ratios obtained during a cooling sequence agree with the established lack of immediate reversibility of the $L_{\beta'}$ to L_βI transition.

The results presented in Figure 2 show that the III/I ratios for PyrPC in 1,2-DPPC are sensitive to the interdigitated phase. It could be argued that the reduced polarity is due to the penetration of ethanol into the bilayer. However, as seen in Figure 2, in the noninterdigitated phase above the main transition temperature, when the lipid is in the L_α phase with and without ethanol, the polarities around the pyrene group are similar with and without ethanol.

The ethanol concentration dependence of the phase changes involving the interdigitated phase is shown in Figure 3A. The temperature of the $L_{\beta'}$ to L_βI transition decreases with increasing concentration of ethanol above 1.2 M. This result agrees in general with our previous results obtained by turbidity and fluorescence measurements (Veiro et al., 1987; Nambi et al., 1988). However, it is interesting that, for the data at 1.2 and 1.4 M ethanol, the maximum polarity achieved above

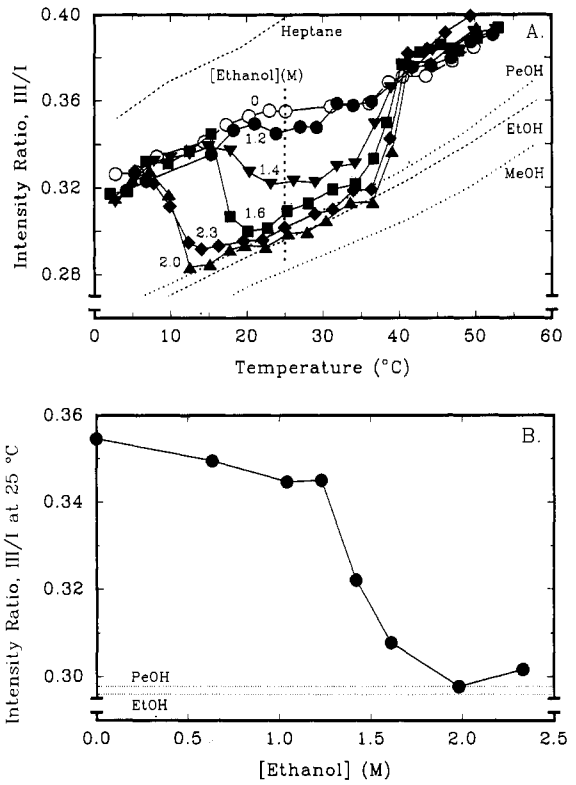

FIGURE 3: (A) III/I ratios for PyrPC upon heating in 1,2-DPPC at various concentrations of ethanol as a function of temperature (symbols and lines). Concentration of ethanol (M): (○) 0; (●) 1.2; (▼) 1.4; (■) 1.6; (▲) 2.0; (◆) 2.3. Lines: see Figure 2. (B) III/I ratios for PyrPC at 25 °C as a function of ethanol concentration (symbols and lines). Lines show the III/I ratio values in organic solvents at 25 °C.

the transition is intermediate between that for the interdigitated and noninterdigitated phases. The III/I ratios at 25 °C as a function of the concentration of ethanol are shown in Figure 3B. The polarity begins to increase from about 1.2 M ethanol and reaches the maximum level of polarity at about 2.0 M ethanol. This suggests that, in the region from 1.2 to 2.0 M ethanol, the L_{β}' or P_{β}' and the $L_{\beta}I$ phases coexist, and above 2.0 M ethanol all of the lipid is in the interdigitated phase. In order to test the possibility that the presence of PyrPC itself had an effect on the induction of interdigitation, an experiment comparing two samples with a 2-fold difference in PyrPC to 1,2-DPPC ratios was performed; no difference in the polarity was found in this experiment.

The behavior of PyrPC in 1,3-DPPC was also studied because it forms the interdigitated phase in the absence of additives (Serrallach et al., 1983). The first heating of 1,3-DPPC after 2 weeks of storage at −3 °C gave a very poor fluorescence intensity, presumably due to the presence of the subgel phase (Serrallach et al., 1983). However, the III/I ratios for PyrPC in 1,3-DPPC on the second heating after a heating–cooling cycle (cooling rate of 3.3 °C per minute) are shown as a function of temperature in Figure 4. The data for 1,2-DPPC from Figure 2 are shown for reference. Below 25 °C the polarity in the vicinity of the pyrene group is slightly higher than that for interdigitated 1,2-DPPC. At about 25 °C there is a small increase in polarity so that the polarity reaches the same level as in the 1,2-DPPC membrane in the presence of 2.0 M ethanol. At about 34 °C, the polarity begins to shift toward less polar values and reaches the same level as in the noninterdigitated phase of 1,2-DPPC at about 36 °C. Serrallach et al. (1983) studied the phase changes of 1,3-DPPC using a combination of X-ray diffraction and DSC measurements and found that a heating run of DSC after an initial

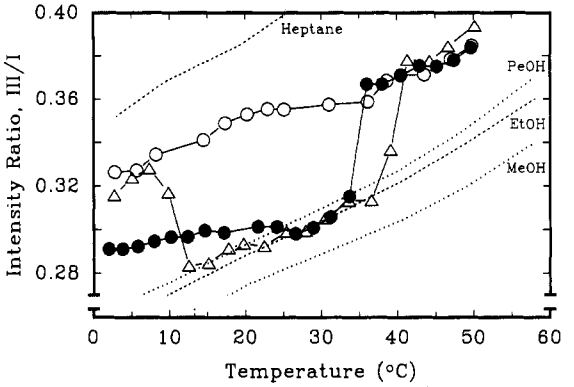

FIGURE 4: III/I ratios for PyrPC upon heating in 1,2- and 1,3-DPPC membranes (symbols and lines): (●) in 1,3-DPPC without ethanol and the second heating run after the heating–cooling cycle; (○) in 1,2-DPPC without ethanol (from Figure 2); (△) in 1,2-DPPC with 2.0 M ethanol (the same data as in Figure 3). Lines: see Figure 2.

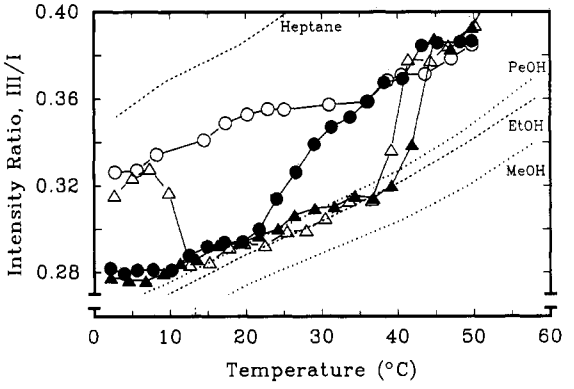

FIGURE 5: III/I ratios for PyrPC upon heating in 1,2-DPPC and DHPC membranes (symbols and lines): (○) in 1,2-DPPC without ethanol (from Figure 2); (△) in 1,2-DPPC with 2.0 M ethanol (from Figure 2); (●) in DHPC without ethanol in an initial heating run after storage at −5 °C for 2 days; (▲) in DHPC with 2.0 M ethanol. Lines: see Figure 2.

heat–cool cycle includes endothermic transitions at 25 and 37 °C, but the phases in each temperature region were not determined. The endothermic transition temperatures agree with the temperatures at which the changes in the III/I ratios for PyrPC were observed in our study. Our results suggest that the $L_{\beta}I$ phase coexists with a noninterdigitated phase below 25 °C and that the 1,3-DPPC membrane is in the $L_{\beta}I$ phase in the region of 25–34 °C. The main transition from $L_{\beta}I$ to L_{α} occurs at 35 °C.

The ether-linked 1,2-dihexadecylphosphatidylcholine (DHPC) also exists in the interdigitated structure in the absence of additives (Ruocco et al., 1985; Laggner et al., 1987). The III/I ratios for PyrPC in DHPC as a function of temperature are shown in Figure 5, along with the data for 1,2-DPPC from Figure 2. In the absence of ethanol, the III/I ratios for DHPC are similar to those for interdigitated 1,2-DPPC in the presence of ethanol up to about 23 °C; the polarity then gradually decreases, reaching the polarity for noninterdigitated lipid at about 36 °C. This corresponds well with the reported transition from $L_{\beta}I$ to P_{β}' at 34.8 °C reported by Ruocco et al. (1985). As is the case with 1,2-DPPC, there is no change in polarity at the main transition temperature since the pyrene moiety remains in the interior of the bilayer during this transition.

The III/I ratios for PyrPC in DHPC in the presence of 2.0 M ethanol are also shown in Figure 5. The environment around the pyrene moiety remains at the characteristic level for interdigitation up to about 40 °C; it then undergoes a rather

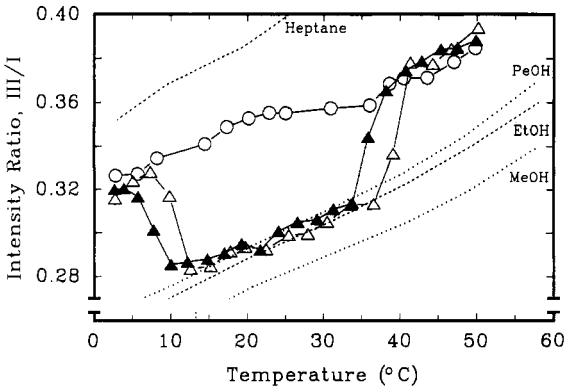

FIGURE 6: III/I ratios for PyrPC upon heating in 1,2-DPPC membranes (symbols and lines): (▲) in 1,2-DPPC with 0.43 M 1-butanol; (○) in 1,2-DPPC without ethanol (from Figure 2); (△) in 1,2-DPPC with 2.0 M ethanol (from Figure 2). Lines: see Figure 2.

steep transition to the characteristic value for a noninterdigitated phase at about 45 °C. Results from this laboratory have previously shown that DHPC undergoes a phase transition from $L_\beta I$ directly to L_α at 47 °C in the presence of 2.0 M ethanol (Veiro et al., 1988). Thus, the observed polarity change agrees with the previously established phase changes.

The results presented thus far for 1,2-DPPC, 1,3-DPPC, and DHPC give similar values of the PyrPC III/I ratio, reflecting similar polarity in the $L_\beta I$ phase in both the presence and absence of ethanol. In order to further investigate whether the polarity observed depends on the presence of ethanol itself or rather is primarily dependent on the lipid structure, we also studied 1,2-DPPC in the presence of 1-butanol, another known inducer of interdigitation in 1,2-DPPC (Herold et al., 1987). The III/I ratios for PyrPC in the 1,2-DPPC membrane in the presence of 0.43 M 1-butanol, where 1,2-DPPC is interdigitated (Herold et al., 1987), are presented as a function of temperature in Figure 6. In the region from about 10 to 35 °C, the polarity around the pyrene group is large, indicating that the 1,2-DPPC is interdigitated in this region, as expected. The polarity in the vicinity of the pyrene group is the same in the $L_\beta I$ region whether butanol or ethanol is the inducer of interdigitation. This suggests that the polarity experienced by the PyrPC in the $L_\beta I$ phase is primarily due to the lipid structure, since it remains the same in the absence of inducer (e.g., in DHPC and 1,3-DPPC) and with either 1-butanol or ethanol as the inducer in 1,2-DPPC.

Fluorescence Intensity III/I Ratios in Cholesterol-Containing Membranes. In Figure 7, the changes in III/I ratios in a 1,2-DPPC membrane containing various amounts of cholesterol with and without ethanol are shown along with the data without cholesterol from Figure 2. The changes in polarity in the region of the $L_\beta I$ phase in the presence of 2.0 M ethanol become less with increasing concentrations of cholesterol, but the temperature regions of the $L_\beta I$ phase are similar to 0 and 5 mol % cholesterol. At 10 mol % cholesterol, the reduction in polarity in the presence of ethanol, representing the $L_\beta I$ phase, is quite small. At 15 mol % cholesterol (not shown), there is a small decrease in polarity in the presence of ethanol. At 25 mol % cholesterol, there is no indication of any interdigitation. The polarity of the lipid in the absence of ethanol decreases slightly with increasing cholesterol concentration. Figure 8 shows the III/I ratios at 25 °C in the presence and absence of ethanol at several cholesterol concentrations. The difference in the III/I ratios with and without ethanol becomes less with increasing cholesterol concentration below 20 mol % cholesterol. The results in Figure 8 show that interdigitation has disappeared by 20 mol % cholesterol. The

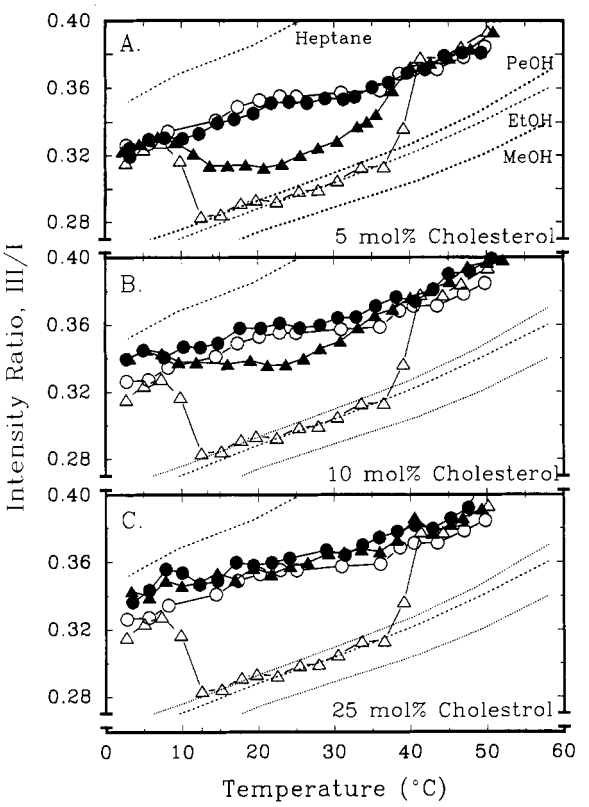

FIGURE 7: III/I ratios for PyrPC upon heating in cholesterol-containing 1,2-DPPC membranes as a function of temperature (symbols and lines): (●) with cholesterol in the absence of ethanol; (▲) with cholesterol in the presence of 2.0 M ethanol; (○) without cholesterol in the absence of ethanol (from Figure 2); (△) without cholesterol in the presence of 2.0 M ethanol (from Figure 2). Cholesterol concentrations (mol %): (A) 5; (B) 10; (C) 25. Lines: see Figure 2.

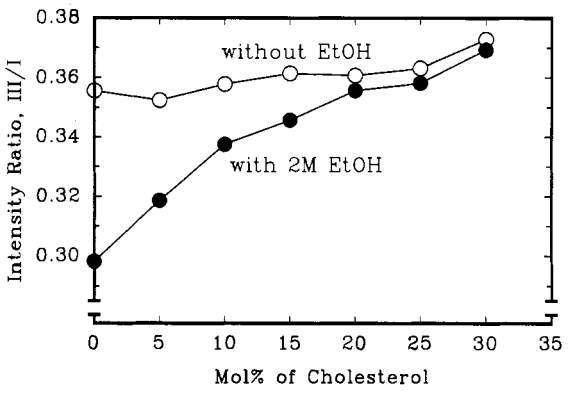

FIGURE 8: III/I ratios for PyrPC at 25 °C in the presence and absence of 2.0 M ethanol as a function of cholesterol concentrations.

slope of the data without ethanol shows the decrease in polarity of the PyrPC environment with increasing cholesterol concentration.

The results shown in Figures 7 and 8 show that cholesterol prevents the induction of interdigitation in 1,2-DPPC by ethanol. They suggest that the fully interdigitated and noninterdigitated phases coexist in the 1,2-DPPC membrane below 20 mol % cholesterol in the presence of ethanol.

The effect of cholesterol on DHPC was also examined. Figure 9 shows the III/I ratios for PyrPC in DHPC containing 20 mol % cholesterol as a function of temperature. DHPC without cholesterol is interdigitated below the pretransition temperature (34.7 °C) without any additives; the data from Figure 5 on DHPC in the absence of cholesterol are shown for comparison in Figure 9. The polarity around the pyrene

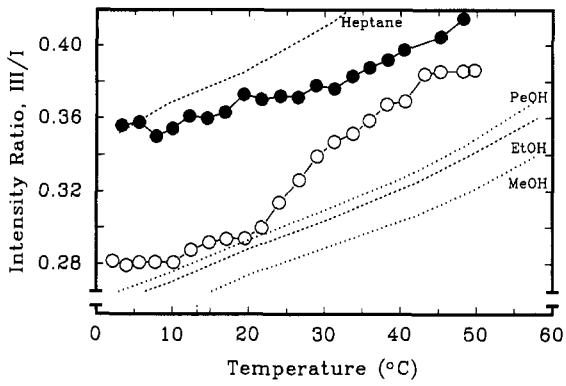

FIGURE 9: III/I ratios for PyrPC upon heating in DHPC with and without cholesterol as a function of temperature (symbols and lines): (○) without cholesterol (the same data as in Figure 5); (●) with 20 mol % cholesterol. Lines: see Figure 2.

group in the DHPC membrane with cholesterol is between that of 1-pentanol and *n*-heptane and agrees well with the polarities observed in the presence of cholesterol for the other lipids in the noninterdigitated phases. These data show that the DHPC membrane containing 20 mol % cholesterol is noninterdigitated at all temperatures. As with the other lipids, the polarity is less in the presence of cholesterol than in its absence, as seen above 40 °C in Figure 9.

Discussion

Fluorescence Methodology. Interdigitation can be detected by X-ray diffraction (Ranck & Tocanne, 1982; Serrallach et al., 1983; McDaniel et al., 1983; McIntosh et al., 1983, 1984; Simon & McIntosh, 1984; Hui et al., 1984; Ruocco et al., 1985; Cunningham & Lis, 1986; Mattai et al., 1987) and neutron diffraction (Braganza & Worcester, 1986). These methods are based on the concomitant decrease in the bilayer thickness and on the increment in the bilayer surface area per head group of lipid. They are important and essential techniques because they are direct methods. However, these methods have a few disadvantages, including the large samples required, the long time required for a measurement in some cases, and the expense of the facilities required.

Spectroscopic techniques such as electron spin resonance (Boggs et al., 1981, 1988, 1989; Boggs & Rangaraj, 1985; Boggs & Mason, 1986), Raman spectroscopy (Levin et al., 1985; Huang et al., 1983; O'Leary & Levin, 1984; Babin et al., 1987), high-pressure infrared spectroscopy (Siminovitch et al., 1987a,b), and ^{31}P and ^{2}H nuclear magnetic resonance (Seelig & Browning, 1978; Tilcock et al., 1986; Xu et al., 1987) have also been used to detect the interdigitated structure in model membranes or biological membranes. The detection mechanisms of these techniques are based upon the fact that the acyl chains of lipids are more motionally restricted and/or ordered in the interdigitated membrane than in the noninterdigitated bilayer.

Fluorescence spectroscopy has been widely utilized to study the structure and dynamics of model membranes and biological membranes. The popularity of the fluorescence technique is due to its sensitivity as well as to the relative simplicity of basic methodology and instrumentation. In the previous paper, we reported the first application of fluorescence spectroscopy to the detection of interdigitation and demonstrated that DPH fluorescence intensity can be used to monitor the noninterdigitated to interdigitated phase transition (Nambi et al., 1988). However, the mechanism of the DPH fluorescence intensity change that accompanies interdigitation has not been established.

Pyrene is another fluorescence probe that has been found to be useful for membrane studies because its vibrational band intensities are sensitive to the polarity of its environment (Nakajima, 1971; Kalyanasundaram & Thomas, 1977; Dong & Winnik, 1984). It has been used to study a variety of micelles and liposomal membranes (Kalyanasundaram & Thomas, 1977; Matsuzaki et al., 1989; Konuk et al., 1989). One problem with pyrene itself is that it has significant water solubility (Galla & Sackmann, 1974; Kalyanasundaram & Thomas, 1977), which can complicate the interpretation of results. Pyrene-labeled lipids have the advantage that they are located in the membrane and the location of the pyrene moiety is known; they have been employed to study several membrane processes such as vesicle fusion (Amselem et al., 1986; Pal et al., 1988; Eklund, 1990).

In the present investigation we have used PyrPC to study the interdigitated phase of several PC's. We have shown that there is a significant change in the intensity ratios of band III (387.5 nm) to band I (376.5 nm) in the transition of these lipids to the interdigitated phase and that the III/I ratio has a characteristic value in the interdigitated phase. The major advantage of this method over our previously reported DPH fluorescence method for studying interdigitation (Nambi et al., 1988) is that the PyrPC measurement not only detects the presence of interdigitation but also gives a measure of the polarity of the environment around the fluorophore. This means that interdigitation can be detected under a particular set of conditions without necessarily detecting a phase change.

The results reported here show that the environment of the pyrene moiety in the $L_{\beta}I$ phase is considerably more polar than in the center of the bilayer, where it is in the L_{β}' phase. However, the polarity observed is not as high as in a true aqueous environment. This suggests that the pyrene moiety is not completely exposed to the aqueous interfacial region but rather is partially submerged in the lipid. The pyrene moiety is connected to the 10-position carbon atom in the 2-acyl chain, and the long axis of the pyrene group turns to the direction perpendicular to the direction of the acyl chains. The 2-pyrenedecanoyl chain is shorter than the 1-palmitoyl chain, suggesting that the pyrene group is not long enough to be directly exposed to the aqueous phase in the interdigitated structure but would be expected to be near the surface, although in a less polar environment than water. This is consistent with our finding that the pyrene group experiences polarities corresponding to ethanol or 1-pentanol in the interdigitated membrane. We also found that the III/I ratios were similar for each of the interdigitated phases, including 1,3-DPPC and DHPC in the absence of inducer, as well as 1,2-DPPC with two different inducers. However, this does not rule out the partial exposure of the pyrene moiety to the interfacial region because the expected amount of alcohol bound in the $L_{\beta}I$ phase is a relatively small mole fraction of the lipid phase (Rowe, 1985b), as is the mole fraction of the fluorophore; it would be expected that a very small proportion of the fluorophores would come in contact with a ligand molecule.

The results obtained in the present study with PyrPC fluorescence are generally in agreement with our previous results using the DPH fluorescence method (Nambi et al., 1988), except for the details of the results at intermediate ethanol concentrations. Although the temperatures of the onset of interdigitation were similar in both studies, the data of Figure 3 suggest that the lipid is only partially interdigitated between 1.2 and 2.0 M ethanol, whereas the X-ray diffraction data from the previous work indicated the 1,2-DPPC was

interdigitated at 1.2 M ethanol. The possibility that PyrPC inhibits interdigitation was ruled out by comparing different PyrPC to 1,2-DPPC ratios. Another possible explanation for this discrepancy that could not be ruled out is that the PyrPC preferentially partitions into regions of the lipid that are in the noninterdigitated phase. This aspect of the methodology warrants further study.

In summary, we have demonstrated that the behavior of the III/I ratios for PyrPC can be used to monitor the presence of interdigitation in PC's. It is unique among the methods used to date in that it reflects the polarity of the environment rather than the degree of order or rate of motion in the acyl chains. In the next section, we will discuss the effects of cholesterol on the transition of 1,2-DPPC to the interdigitated structure in the presence of ethanol as determined by using this technique.

Effects of Cholesterol on the Interdigitated Gel Phase. We have shown that 20 mol % cholesterol prevents the induction of interdigitation by ethanol in 1,2-DPPC. Many substances are known to induce the formation of the interdigitated structure, and the mechanisms have been discussed by several authors (Simon & McIntosh, 1984; Simon et al., 1986; Nambi et al., 1988; Rowe & Cutrera, 1990). The interdigitated phase is characterized by a greater area per head group, which alleviates the head-group crowding in PC's due to the discrepancy between the cross section of the head groups and the acyl chains, and by a closer van der Waals packing in the acyl-chain region. The unfavorable effect on the exposure of the terminal methyl groups to the aqueous interfacial region is alleviated by the binding of the amphiphilic inducer molecules, which are less polar than the water they replace. From the point of view of these considerations, cholesterol may prevent interdigitation by increasing the area per head group in the gel phase, thereby eliminating head-group crowding, which is one of the factors favoring interdigitation.

Our data for cholesterol contents below 20 mol % are consistent with the model of cholesterol/1,2-DPPC interactions suggested by Copeland and McConnell (1980) in their study of this binary system. They suggested that below 20 mol % cholesterol there are two phases present, one that is 20 mol % cholesterol and the other that is pure 1,2-DPPC. Our results, shown in Figure 8, in which the onset of interdigitation was not affected but the degree of polarity change in the $L_{\beta}I$ phase becomes smaller with increasing cholesterol, are consistent with this model, assuming the regions of pure 1,2-DPPC become interdigitated and the regions containing 20 mol % cholesterol do not.

Cholesterol also prevents interdigitation in the ether-linked DHPC, as was shown for 50 mol % cholesterol by Siminovich et al. (1987a) and in the present study for 20 mol % cholesterol. The major difference between the ester- and ether-linked lipids is a conformational difference at the glycerol backbone as a result of replacing the ester sp^2 carbon of the carbonyl group with an sp^3 carbon of the alkyl hydrocarbon chain (Ruocco et al., 1985). This leads to a mismatch of the ester-linked molecules between the head-group and alkyl-chain cross sections, which apparently contributes to interdigitation. It was previously suggested that cholesterol eliminates interdigitation in DHPC because it occupies a location near the interface, increasing the area per head group and reducing the mismatch (Siminovich et al., 1987a). Thus the mechanism of the elimination of interdigitation by cholesterol in DHPC is probably similar to its role in preventing interdigitation in 1,2-DPPC.

Chong and Choate (1989) studied the effects of cholesterol on the phase transition of the asymmetric mixed-chain phosphatidylcholine, 1-stearoyl-2-caproyl-*sn*-glycero-3-phosphocholine, using DSC. Kao et al. (1990) used both DSC and fluorescence to study the behavior of dehydroergosterol in interdigitated lipid membranes. They found that cholesterol and dehydroergosterol cause a disordering effect on the packing of the chains in the mixed interdigitated structure, but they could not determine if the structure remained interdigitated in the presence of the sterols. Our results support their idea that sterols may have a function in preventing lipids from forming highly ordered interdigitated structures in membranes.

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Epitope Mapping of Antibodies to Acetylcholine Receptor α Subunits Using Peptides Synthesized on Polypropylene Pegs†

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ABSTRACT: Concurrent synthesis of overlapping octameric peptides corresponding to the sequence of the *Torpedo* acetylcholine receptor (AChR) α subunit has been carried out on polypropylene supports functionalized with primary amino groups according to a method developed by M. Geysen [(1987) *J. Immunol. Methods 102*, 259-274]. The peptides on the solid supports have been used in an enzyme-linked immunosorbent assay. Interactions of the synthetic peptides with antibodies are then detected without removing them from the solid support. By this procedure, epitopes of both antisera and monoclonal antibodies to the *Torpedo* acetylcholine receptor, its subunits, and synthetic peptide fragments have been mapped. Both rat and rabbit antisera to the α subunit show major epitopes spanning the residues 150-165, 338-345, and 355-366 on the *Torpedo* AChR α subunit. Epitopes of monoclonal antibodies to these major epitopes and to others have been rather precisely mapped by using this technique with peptides of varying lengths. The specificity of several of these mAbs are of interest because they have been used in mapping the transmembrane orientation of the AChR α-subunit polypeptide chain.

Mapping epitopes on the primary sequence of subunits of nicotinic acetylcholine receptors (AChRs)[1] is important because monoclonal antibodies (mAbs) have proven to be useful probes for AChR structure and because autoantibodies to muscle AChRs are responsible for causing the muscular weakness characteristics of myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG).

AChRs of fish electric organs and mammalian muscle are composed of four types of homologous subunits arranged like

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[1] Abbreviations: αBgt, α-bungarotoxin; ABTS, 2,2′-azinobis[3-ethylbenzothiazolinesulfonate] diammonium salt; AChR, acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; ELISA, enzyme-linked immunosorbent assay; Fmoc-aa-OPfp, (fluorenylmethyloxycarbonyl)amino acid pentafluorophenyl ester; mAb, monoclonal antibody; MAR, mouse anti-rat IgG; MG, myasthenia gravis; MIR, main immunogenic region; PBS, phosphate-buffered saline.

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