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Influence of Cholesterol on the Structural Preferences of Dioleoylphosphatidylethanolamine–Dioleoylphosphatidylcholine Systems: A Phosphorus-31 and Deuterium Nuclear Magnetic Resonance Study†

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ABSTRACT: The polymorphic phase behavior of mixtures of synthetic dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) and the influence of cholesterol on these phase preferences have been investigated by employing nuclear magnetic resonance (NMR) techniques. In particular, ^{31}P NMR procedures are utilized to study the overall phase preferences of these mixed systems, whereas ^{2}H NMR is employed to monitor the structural preferences of individual components of these systems by using versions of DOPE and DOPC which are deuterium (^{2}H) labeled at the C_{11} position of the acyl chains. The results obtained show that DOPE–DOPC systems containing as little as 20 mol % DOPC initially assume lamellar structure at 40 °C, even though DOPE in isolation prefers the hexagonal (H_{II}) organization at this temperature. However, this lamellar organization appears to represent a metastable state, as incubation for extended periods at 40 °C results in formation of a structure, possibly the cubic phase, in which the phospholipids experience isotropic motional averaging. The addition of cholesterol induces hexagonal (H_{II}) phase organization. ^{2}H NMR studies of appropriately labeled versions of these systems indicate that cholesterol does not produce such effects by associating preferentially with either DOPE or DOPC. Further, in situations where bilayer, hexagonal, or "isotropic" phases coexist in the same sample, the phospholipids exhibit apparently ideal mixing behavior.

The functional roles of cholesterol in biological membranes are not well understood. Within the terms of the fluid mosaic model (Singer & Nicholson, 1972), it is generally postulated that cholesterol plays a role in modulating the "fluidity" of the lipid environment, thereby affecting membrane function. However, there is little evidence to support the proposal that modulation of fluidity plays important functional roles in vivo. Reasons for this include the fact that lipids such as gel-state lipids which can inhibit the activity of integral protein in reconstituted protein–lipid systems [see, for example, Warren et al. (1975)] do not appear to exist in most biological systems. This is particularly true of eukaryotic cell membranes. Further, the ability of physiologically relevant factors (such as pH, ionic strength, divalent cation concentration, or even membrane protein) to isothermally modulate membrane fluidity to gain the necessary regulation in appropriate lipid mixtures is far from established.

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In this context, it is appropriate to examine the influence of cholesterol on physical properties of membrane phospholipids other than fluidity. In previous work (Cullis & de Kruijff, 1979; Cullis et al., 1978), we have shown that cholesterol exerts a rather remarkable effect in unsaturated phosphatidylcholine (PC)–phosphatidylethanolamine (PE) systems, serving to destabilize bilayer structure and induce formation of the hexagonal (H_{II}) phase. In 18:1_c/18:1_c PE (DOPE)[1]–18:1_c/18:1_c PC (DOPC) (1:1) systems, for example, cholesterol induces apparently complete H_{II} phase structuring at equimolar proportions with respect to phospholipid (Cullis et al., 1978). This result is surprising in view of the well-known ability of cholesterol to "condense" PC monolayers and reduce membrane permeability (Demel & de Kruijff, 1976) and thus in some sense stabilize bilayer structure for PC systems.

In the present work, we examine in somewhat greater detail the structural preferences of DOPC–DOPE systems and the influence of cholesterol on these preferences in the hope of answering the following questions. First, what are the minimum DOPC concentrations required to stabilize DOPE in a bilayer organization, and what intermediary structures are obtained at lower DOPC concentrations? Second, what are the effects of cholesterol on systems composed of DOPC–DOPE at various ratios, and is there any evidence of a preferential association of cholesterol with either lipid species? Third, in DOPE–DOPC–cholesterol systems exhibiting both bilayer and H_{II} phase structure, is there any segregation of either phospholipid species into a particular phase, or do the lipids exhibit ideal mixing? We show that as little as 20 mol % DOPC can briefly stabilize a bilayer organization for DOPE at 40 °C but that prolonged incubation (1 h) leads to formation of a new phase possibly of cubic (Wieslander et al., 1981) structure. In such systems, the presence of as little as 2 mol % cholesterol (with respect to total phospholipid) leads to relatively immediate formation of H_{II} phase organization, with no evidence for a preferential association of cholesterol with either lipid species. Finally, in mixed liquid-crystalline DOPC–DOPE–cholesterol systems exhibiting both bilayer and H_{II} phase characteristics, the lipids appear to be equally distributed in both phases.

Materials and Methods

Lipid Synthesis. Chromatographically pure oleic acid, dimethyl sulfoxide (Me_2SO), and 1,1′-carbonyldiimidazole were obtained through Sigma (St. Louis, MO). Tetrabutylammonium hydroxide (25% in methanol) was obtained from Eastman-Kodak (Rochester, NY). Tetrahydrofuran, azelaic acid, and nonanol were obtained from Aldrich. Other reagents were of analytical grade. Chloroform and methanol were distilled immediately before use. 11,11-Dideuteriooleic acid was synthesized by a modification of the procedure of Tucker et al. (1971), based on a Wittig condensation of nonanol-2-d_2 with methyl 9-iodononanoate (S. B. Farren and P. R. Cullis, unpublished experiments). DOPC and DOPE (both deuterated and nondeuterated) were synthesized by the procedure of Warner & Benson (1977) and purified as previously described (Tilcock & Cullis, 1982).

Nuclear Magnetic Resonance. ^2H NMR and ^{31}P NMR spectra were obtained by using a Bruker WP200 spectrometer operating at 30.7 MHz for ^2H and 81 MHz for ^{31}P. Lipid mixtures were dispersed by vortexing in 10 mM Tris-HCl, 100 mM NaCl, 100 mM NaCl, and 2 mM EDTA, pH 7, which, in the case of the ^2H-labeled lipids, was prepared in deuterium-depleted water. For ^{31}P NMR, free induction decays were accumulated from up to 1000 transients by employing a 15-μs 90° radio-frequency pulse, 20-kHz sweep width, and 0.8-s interpulse delay, in the presence of broad-band proton decoupling. An exponential multiplication corresponding to 50-Hz line broadening was applied to the free induction decay prior to Fourier transformation.

For ^2H NMR, free induction decays were accumulated for up to 20 000 transients by employing a 15-μs 90° radio-frequency pulse, 30-kHz sweep width, and 0.04-s interpulse delay. An exponential multiplication corresponding to a 100-Hz line broadening was applied prior to Fourier transformation. It may be noted that due to "dead-time" limitations, the broader ^2H NMR spectra obtained are distorted. In particular, the classic "solid-state" ^2H NMR pattern (see the H_{II} phase ^2H NMR spectrum of [C_{11}-$^2H_2$]DOPE in Figure 1) is not observed for broader "bilayer" ^2H NMR spectra (see the bilayer [C_{11}-$^2H_2$]DOPE spectrum at 0 °C, Figure 1). In agreement with other workers (Brown & Seelig, 1977) subject to similar instrumental limitations, two peaks corresponding to the two major peaks of the solid-state pattern are observed, but other detail is lost.

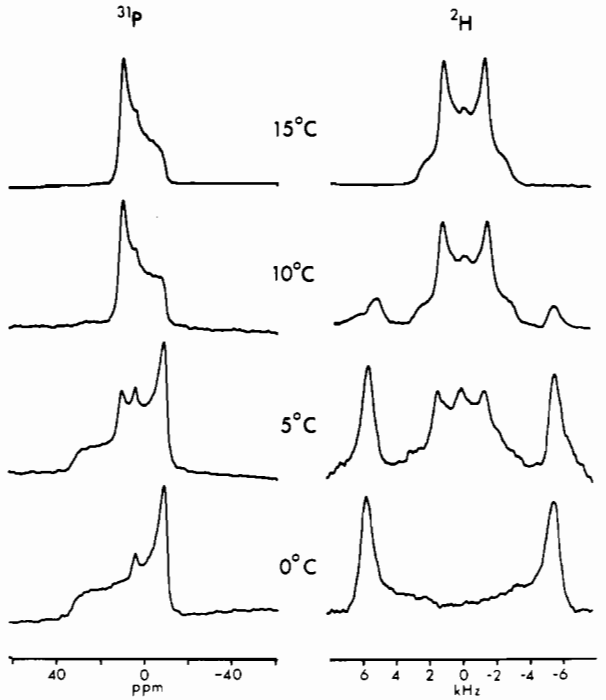

FIGURE 1: ^{31}P and ^2H NMR spectra as a function of temperature of fully hydrated dioleoylphosphatidylethanolamine (DOPE) which is ^2H labeled at the C_{11} position of the acyl chains ([C_{11}-$^2H_2$]DOPE). The ^{31}P NMR spectra were obtained at 81.0 MHz in the presence of proton decoupling, whereas the ^2H NMR spectra were obtained at 30.7 MHz. For details, see Materials and Methods.

Results

As has been well discussed elsewhere [for a review, see Cullis & de Kruijff (1979)], ^{31}P NMR can be usefully employed to determine the bilayer or hexagonal (H_{II}) organization of hydrated phospholipid systems. A disadvantage of this technique, however, is that in mixed phospholipid systems the structural preferences of individual species in the mixture cannot be resolved. A method of overcoming such difficulties is inherent in the observation that ^2H NMR studies on ^2H-labeled lipids

[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine; [C_{11}-$^2H_2$]DOPC, 1,2-(11,11-dideuteriodioleoyl)-*sn*-glycero-3-phosphorylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphorylethanolamine; [C_{11}-$^2H_2$]DOPE, 1,2-(11,11-dideuteriodioleoyl)-*sn*-glycero-3-phosphorylethanolamine; NMR, nuclear magnetic resonance; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

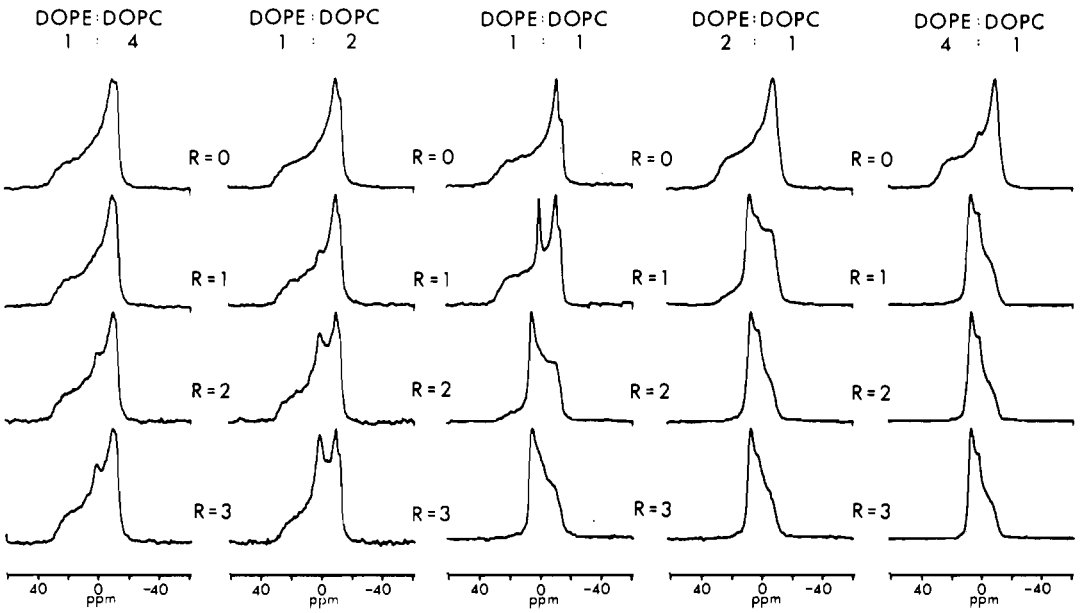

FIGURE 2: 81.0-MHz ^{31}P NMR spectra at 40 °C of aqueous dispersions of mixtures of dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) at various ratios in the presence of varying amounts of cholesterol. The ratio R refers to the molar ratio of cholesterol to DOPC contained in the sample. For details of sample preparation and data accumulation, see Materials and Methods.

can also yield information on the bilayer or H_{II} phase preferences (Gally et al., 1980; Cullis et al., 1981). The essence of the technique is illustrated in Figure 1 for DOPE which is ^2H labeled at the C_{11} position ([C_{11}-$^2H_2$]DOPE). The ^{31}P NMR spectra illustrate a bilayer to hexagonal (H_{II}) transition occurring as the temperature is raised through 10 °C as indicated by the change in line shape from one with a low-field shoulder and high-field peak separated by ~50 ppm (bilayer spectrum) to a spectrum with reversed asymmetry which is a factor of 2 narrower (hexagonal H_{II} spectrum). This behavior is very similar to that observed for unlabeled DOPE (Cullis & de Kruijff, 1976; Tilcock & Cullis, 1982). This transition is reflected in the corresponding ^2H NMR spectra by the appearance of a narrower solid-state pattern arising from H_{II} phase [C_{11}-$^2H_2$]DOPE as the temperature is raised. It may be noted that theory predicts a reduction of the quadrupolar splitting (ΔQ, the frequency separation between the major peaks of the ^2H spectra) by a factor of 2 on proceeding from the bilayer to the H_{II} phase (Gally et al., 1980). The results of Figure 1 show a somewhat larger reduction in ΔQ for [C_{11}-$^2H_2$]DOPE (from 11.3 to 2.7 kHz), indicating appreciably less local order at the C_{11} position for DOPE in the H_{II} organization.

A first objective of this study was to further characterize the influence of cholesterol on mixed DOPE–DOPC systems of varying composition. As illustrated in Figure 2, 20 mol % or more DOPC in DOPE appears sufficient (but see Figure 3) to induce a net bilayer organization at 40 °C. It may be observed that progressively more cholesterol is required to induce H_{II} phase structure as the DOPC content is increased. For the DOPE–DOPC (4:1) systems, a cholesterol to DOPC molar ratio (R) of 1 (corresponding to 17 mol % cholesterol with respect to total lipid) induces relatively complete H_{II} phase organization, whereas R = 2.0 (40 mol % cholesterol) was required for the DOPE–DOPC (2:1) system and R = 3.0 (60 mol % cholesterol) for the DOPE–DOPC (1:1) systems. Systems containing higher amounts of DOPC such as DOPE–DOPC (1:2) do not adopt H_{II} phase structure even for cholesterol contents as high as R = 3.0, which corresponds to a 67 mol % cholesterol content, although some evidence of a narrow resonance which appears to occur as a precursor to

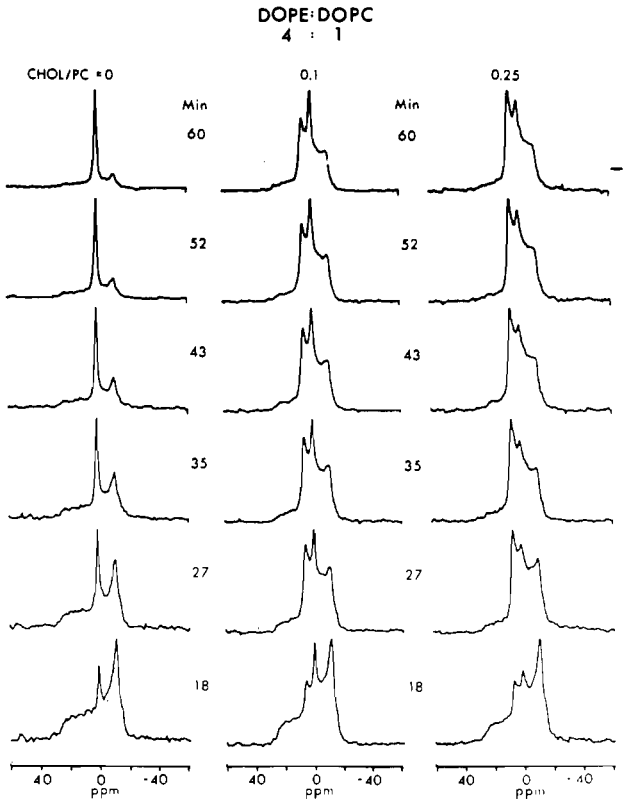

FIGURE 3: Time dependence of 81.0-MHz ^{31}P NMR spectra at 40 °C arising from aqueous dispersions of dioleoylphosphatidylethanolamine (DOPE)–dioleoylphosphatidylcholine (DOPC) at a DOPE:DOPC molar ratio of 4:1 and in the presence of varying amounts of cholesterol. The ratio CHOL/DOPC refers to the molar ratio of cholesterol to DOPC. The samples were hydrated at 20 °C, and spectra were collected sequentially at 40 °C in accumulations lasting 10 min each after an initial temperature equilibration period of 10 min. For other details of sample preparation and data manipulation, see Materials and Methods.

H_{II} phase organization is observed at these higher cholesterol levels.

Previous studies (Cullis & de Kruijff, 1978) have shown that concentrations of (soya) PC in (soya) PE below those required

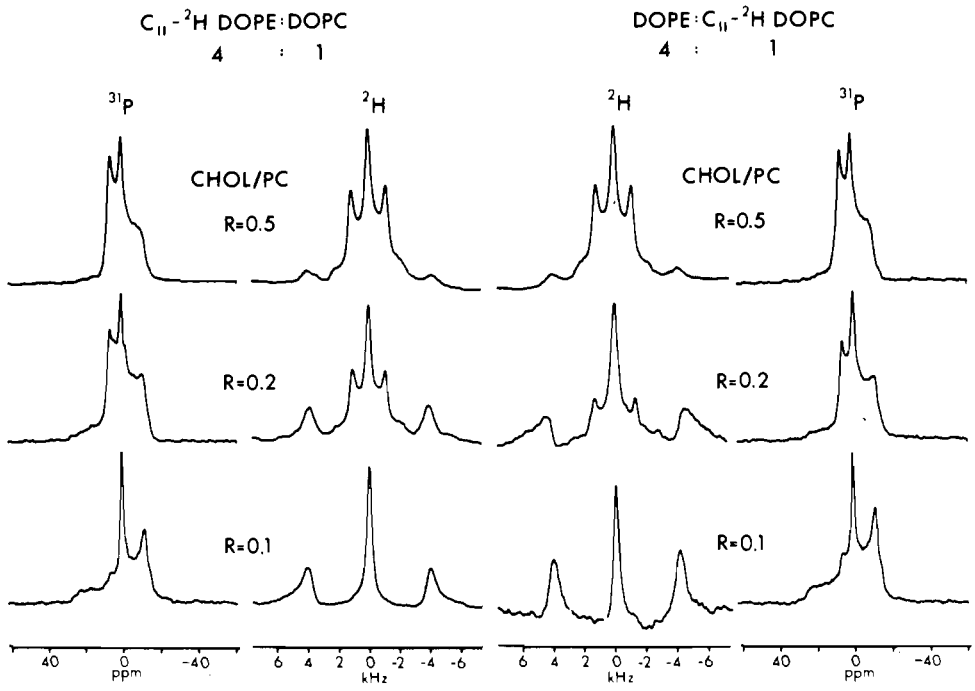

FIGURE 4: 81.0-MHz ^{31}P NMR and 30.7-MHz ^{2}H NMR spectra at 40 °C arising from aqueous disperions of mixtures of dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) and cholesterol (CHOL) at a DOPE:DOPC molar ratio of 4:1 where either the DOPE is ^{2}H labeled at the C_{11} position ([C_{11}-$^2H_2$]DOPE) or the DOPC is ^{2}H labeled at the C_{11} position ([C_{11}-$^2H_2$]DOPC). The ratio R refers to the molar ratio of cholesterol to DOPC. For details of sample preparation and data manipulation, see Materials and Methods.

to induce stable lamellar systems often give rise to systems which exhibit a narrow isotropic ^{31}P NMR signal characteristic of structures which allow isotropic motional averaging. Similar behavior was observed for DOPC–DOPE systems at 40 °C where the DOPC comprised less than 20 mol % of the phospholipid. The ^{31}P NMR spectra obtained were highly variable, however, and it was finally ascertained that such DOPC–DOPE systems undergo time-dependent structural transitions. This time-dependent evolution was also apparent for the DOPE–DOPC (4:1) system as shown in Figure 3. In agreement with the results of Figure 2, in the first 10-min accumulation, a purely bilayer ^{31}P NMR spectrum was obtained; however, at longer incubation times, a narrow spectral component becomes increasingly dominant. In concert with this evolution, an increasing part of the aggregated lipid dispersion becomes optically translucent. The structure of this translucent phase is not known; however, other authors (Luzzatti et al., 1968; Wieslander et al., 1981) have identified lipid structures with similar optical characteristics as having a cubic structure. An unequivocal demonstration of such a structure in the DOPE–DOPC (4:1) systems awaits X-ray studies; however, such a phase would be consistent with the presence of isotropic motional averaging for the ^{31}P NMR spectra. Also, the long equilibration time to effect the transformation would be consistent with a conversion between the essentially two-dimensional lamellar structure to the intercalated lipid cylinders of the cubic phase which has three-dimensional characteristics. It may be noted that this behavior was entirely reversible as freeze–thawing the sample caused a reversion to a milky appearance characteristic of liposomal dispersions, and subsequent incubation at 40 °C again resulted in the progression noted in Figure 3.

The addition of cholesterol to the DOPE–DOPC (4:1) sample to achieve a cholesterol to DOPC ratio of 0.1 (corresponding to a cholesterol content of 2 mol %) has strong effects as also shown in Figure 3. In particular, the presence of this very small amount of cholesterol results in significant H_{II} phase formation after 10 min of incubation at 40 °C. Higher cholesterol levels corresponding to $R = 0.25$ (5 mol % cholesterol) cause a larger H_{II} phase component and reduced equilibration times to reach an equilibrium situation. For both $R = 0.1$ and 0.25, an isotropic component appears at extended incubation times, but the intensity of this resonance is much reduced by the presence of cholesterol. It may be noted that the spectra obtained after 1 h appear to represent an equilibrium situation as no further changes for longer incubations (2 h) were observed. These results show that the presence of cholesterol mitigates against formation of the phase allowing isotropic motion and engenders H_{II} phase structure.

The mechanism whereby cholesterol promotes H_{II} phase formation in mixed PE–PC systems is not understood. One possibility would be that it associates preferentially with the bilayer-stabilizing PC species [see van Dijck et al. (1976)] and that the resulting PC–cholesterol complex is more readily incorporated into the H_{II} phase matrix. Alternatively, it is conceivable that cholesterol induces a lateral segregation of PC, allowing the PE to adopt the H_{II} phase it prefers in isolation. In order to investigate these possibilities, we employed the ^{2}H-labeled [C_{11}-$^2H_2$]DOPE and [C_{11}-$^2H_2$]DOPC. The ^{31}P and ^{2}H NMR results obtained from [C_{11}-$^2H_2$]DOPE–DOPC (4:1) and DOPE–[C_{11}-$^2H_2$]DOPC (4:1) (at 40 °C) in the presence of varying amounts of cholesterol are shown in Figure 4. There are several features of interest. First, the ^{31}P NMR spectra for mixtures containing ^{2}H-labeled DOPC or DOPE are virtually identical, both with each other and with the corresponding mixtures containing unlabeled lipids (Figure 3). Second, the ^{2}H NMR spectra arising from [C_{11}-$^2H_2$]-DOPE or [C_{11}-$^2H_2$]DOPC in mixtures containing the same proportions of PE, PC, and cholesterol are also very nearly identical. Thus, DOPC and DOPE appear to partition with equal probability between the various phases (bilayer, H_{II}, or isotropic) present in the sample. This result demonstrating miscibility of (liquid-crystalline) DOPE and DOPC even when different phase structures are present is rather surprising as

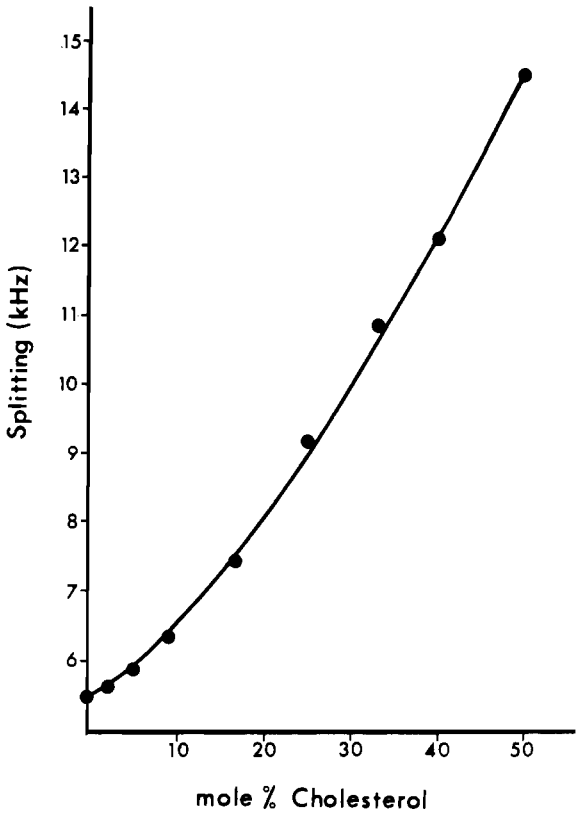

FIGURE 5: Influence of cholesterol on the quadrupolar splitting ΔQ observed for dioleoylphosphatidylcholine (DOPC) ^2H labeled at the C_{11} position of the acyl chains ([C_{11}-$^2H_2$]DOPC). The ^2H NMR spectra from which these data were taken were obtained at 30.7 MHz and at a temperature of 40 °C. For details of sample preparation and NMR data manipulation, see Materials and Methods.

it may have been expected that DOPC would be present at higher levels in the bilayer phase component, and DOPE in the H_{II} phase component.

A final point of interest regarding the ^2H NMR spectra of Figure 4 concerns the values of quadrupolar splitting observed. First, the quadrupolar splittings (ΔQ) of the [C_{11}-$^2H_2$]DOPE and [C_{11}-$^2H_2$]DOPC are equivalent in both the bilayer and H_{II} organization. It may be noted that for the cholesterol contents corresponding to $R = 0.1$, the maximum ΔQ expected for the [C_{11}-$^2H_2$]DOPC (if all the cholesterol was associated with the DOPC) would be 6.3 kHz (see Figure 5). The value of 8.1 kHz observed both for the labeled DOPC and for DOPE indicates that bilayer phase DOPE has a more ordered hydrocarbon region than DOPC and that this increased order is also experienced by the acyl chains of the DOPC. This is again consistent with a well-mixed system. Second, even in the presence of the highest cholesterol levels used ($R = 0.25$), the ΔQ of the bilayer component of the labeled DOPC sample remains at 8.3 kHz. If the cholesterol present was preferentially associated with the PC, the results of Figure 5 suggest that the ΔQ should be increased by almost 3 kHz. Thus, the results of Figure 4 give no support for the notion of a preferential association of cholesterol with PC over PE.

Discussion

The results of this investigation significantly extend our understanding of the structural properties of mixtures of unsaturated PC–PE systems and the influence of cholesterol on those properties. We would like to draw attention to three aspects of these results. The first of these concerns the narrow ^{31}P NMR spectral component observed for the DOPE–DOPC (4:1) systems. Such a spectral component is a ubiquitous feature of ^{31}P NMR spectra obtained from mixed lipid systems containing bilayer and H_{II} phase lipids (Cullis & de Kruijff, 1978; Cullis et al., 1978; Tilcock & Cullis, 1981; de Kruijff et al., 1979) and has been attributed to lipidic particles (inverted micelles) (Verkleij et al., 1979), a honeycomb structure (Cullis et al., 1980), small bilayer vesicles [see Nayar et al. (1982)], or alternative structures such as the cubic (Luzzatti et al., 1968) or rhombic (Luzzatti et al., 1969) phases. While our results do not establish unambiguously the nature of this structure for DOPE–DOPC mixtures, they do establish that the bilayer organization observed at low (e.g., 20 mol % or less) PC concentrations is a metastable state at 40 °C and is not the equilibrium state. In particular, it is clear that there is a high potential for confusion in these studies, and a detailed examination of the time-dependent behavior is necessary before unambiguous information regarding the equilibrium organization of component lipids is available. Thus, our initial question regarding the minimum DOPC concentrations required to stabilize DOPE in a bilayer organization must remain unanswered, as such concentrations will be sensitive to the temperature of observation and the length of equilibration time at that temperature. Concentrations of DOPC greater than 20 mol % result in bilayer stabilization at 40 °C for times on the order of hours or longer; however, more prolonged incubation periods may well result in structural alterations. Such studies were not attempted here due to problems involving lipid degradation which necessarily accompany long incubations at elevated temperatures.

The second point of discussion concerns the ability of cholesterol to induce H_{II} phase structure in mixed unsaturated PE–PC systems. We do not presently understand the mechanism whereby cholesterol induces such effects. The results presented here suggest that cholesterol is not preferentially associated with the PC component, which argues against the possibility that cholesterol is associated with PC to form a "cone"-shaped [see Cullis & de Kruijff (1979)] complex more compatible with the H_{II} phase. Similarly, the results argue against explanations involving segregation of different lipid species into different phase structures. A remaining possibility is that the distributed presence of the cone-shaped cholesterol molecule promotes the transition to the H_{II} phase. This is unlikely to be a complete explanation, however, as the effect of introducing more of the cone-shaped PE molecules to the DOPE–DOPC (4:1) systems results in a greater tendency to adopt the phase characterized by isotropic motional averaging. It may be that the influence of cholesterol depends to some extent on the metastable nature of the PE–PC system itself. It is, for example, clear that the presence of relatively small amounts of PC inhibits formation of the H_{II} phase even though this phase is the lowest energy configuration for the large majority of the endogenous lipid. The reasons for this are not obvious; however, we have noted elsewhere (Cullis et al., 1980) that the bilayer–hexagonal transition for unsaturated PE appears to proceed as an interbilayer event, and thus any factors which mitigate against close apposition of bilayers will inhibit such transitions. X-ray studies (Philips et al., 1972) have shown that the interlamellar repeat distances for (saturated) PE's are appreciably smaller than those for corresponding PC's, a feature which has been attributed to the limited hydration of the PE head group in comparison to PC. It is therefore possible that the distributed presence of minority PC components in mixtures with PE increases the interlamellar repeat distance to the extent that H_{II} phase formation is not possible, resulting in production of other structures such as the cubic phase. Conversely, the introduction of relatively

small amounts of cholesterol (which hydrates very poorly in comparison to phospholipids) could reduce the interbilayer separation to the extent that H_{II} phase formation is again favored. A resolution of such possibilities awaits detailed X-ray studies on these mixed lipid systems.

The final point of discussion concerns the observation that in mixed DOPE–DOPC–cholesterol systems which exhibit regions of bilayer and H_{II} phase organization (as well as structure allowing isotropic motional averaging) the DOPC and DOPE appear to partition into these structures with equal probability. The resulting conclusion that these lipids remain ideally mixed even when such diverse structural alternatives are available to them suggests that they will also behave in a miscible fashion in less extreme situations. In particular, it has been argued that local clusters with differing lipid compositions from the surrounding bulk lipid may exist in biological membranes and that these microenvironments may modulate local function. The results presented here would argue against such a hypothesis, suggesting that such clusters would not exist for times longer than the NMR time scale ($\sim 10^{-5}$ s).

In summary, the results presented here lead to the following conclusions. First, the observed phase behavior of mixtures of bilayer and H_{II} phase lipid can represent metastable states and not the equilibrium organization. Second, the presence of cholesterol strongly facilitates formation of H_{II} phase structure in DOPE–DOPC systems and inhibits formation of other structures, possibly cubic in nature, which allow isotropic motional averaging on the NMR time scale. Finally, in mixed liquid-crystalline PC–PE systems, the lipids remain ideally mixed even when different phase structures coexist.

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