Studies of Mixed-Chain Diacyl Phosphatidylcholines with Highly Asymmetric Acyl Chains: A Fourier Transform Infrared Spectroscopic Study of Interfacial Hydration and Hydrocarbon Chain Packing in the Mixed Interdigitated Gel Phase

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ABSTRACT The mixed interdigitated gel phases of unlabeled, specifically ^{13}C=O-labeled, and specifically chain-perdeuterated samples of 1-*O*-eicosanoyl, 2-*O*-lauroyl phosphatidylcholine and 1-*O*-decanoyl, 2-*O*-docosanoyl phosphatidylcholine were studied by infrared spectroscopy. Our results suggest that at the liquid-crystalline/gel phase transition temperatures of these lipids, there is a greater redistribution in the populations of free and hydrogen-bonded ester carbonyl groups than is commonly observed with symmetric chain *n*-saturated diacyl phosphatidylcholines. The formation of the mixed interdigitated gel phase coincides with the appearance of a marked asymmetry in the contours of the C=O stretching band, a process which becomes more pronounced as the temperature is reduced. This asymmetry is ascribed to the emergence of a predominant lipid population consisting of free *sn*1- and hydrogen-bonded (hydrated) *sn*2-ester carbonyl groups. This suggests that the region of the mixed interdigitated bilayer polar/apolar interface near to the *sn*1-ester carbonyl group is less hydrated than is the case with the noninterdigitated gel-phase bilayers formed by normal symmetric chain phosphatidylcholines. In the methylene deformation region of the spectrum, the unlabeled lipids exhibit a pronounced splitting of the CH_2 scissoring bands. This splitting is significantly attenuated when the short chains are perdeuterated and collapses completely upon perdeuteration of the long chains, irrespective of whether the long (or short) chains are esterified to the *sn*1 or *sn*2 positions of the glycerol backbone. These results are consistent with a global hydrocarbon chain packing motif in which the zigzag planes of the hydrocarbon chains are perpendicular to each other and the sites occupied by long chains are twice as numerous as those occupied by short chains. The experimental support for this chain-packing motif enabled more detailed considerations of the possible ways in which these lipid molecules are assembled in the mixed interdigitated gel phase. Generally, our results are compatible with a previously proposed model in which the mixed interdigitated gel phase is an assembly of repeat units which consists of two phosphatidylcholine molecules forming a triple-chain structure with the long chains traversing the bilayer and with the methyl termini of the shorter chains opposed at the bilayer center. Our data also suggest that the packing format which is most consistent with our results and previously published work is one in which the hydrocarbon chains of each repeat unit are parallel to each other with the repeat units themselves being perpendicularly packed.

INTRODUCTION

The physical properties of mixed chain PCs[1] have been the focus of increasingly intensive study because diacylglycerolipids containing two different hydrocarbon chains are the dominant molecular species found in the overwhelming majority of natural cell membranes (for reviews, see Huang and Mason (1986) and Huang (1990)). Mixed chain PCs containing two *n*-saturated fatty acyl chains have been most widely studied primarily by DSC (Keough and Davis, 1979; Chen and Sturtevant, 1981; Stümpel et al., 1981; Mason et al., 1981; Serralach et al., 1984; Xu and Huang, 1987; Xu et al., 1987; Mattai et al., 1987; Shah et al., 1990; Lin et al., 1990; Wang et al., 1990; Blutmann et al., 1991), ^{31}P and ^{13}C NMR spectroscopy (Lewis et al., 1984; Xu et al., 1987), x-ray diffraction (McIntosh et al., 1984; Hui et al., 1984; Mattai et al., 1987; Shah et al., 1990), and vibrational spectroscopy (Wong and Huang, 1989; Huang et al., 1983; Slater et al., 1992). Such studies have identified an unusual and interesting class of mixed chain PCs in which the effective length of one of acyl chain is approximately twice the length of the other (McIntosh et al., 1984; Hui et al., 1984; Huang and Mason, 1986; Xu and Huang, 1987; Xu et al., 1987; Mattai et al., 1987; Wong et al., 1989; Huang, 1990; Shah et al., 1990; Slater et al., 1992). DSC studies have shown that the thermodynamic properties of hydrated lipid bilayers composed of this particular class of mixed chain PCs are discontinuous from trends extrapolated from similar studies of the symmetric chain PCs and the other mixed chain PCs that have been studied so far (see Huang and Mason, 1986; Huang, 1990, 1991; Marsh, 1992). Such observations led to the suggestion that these lipids probably adopt an unusual packing motif in the gel phase, a suggestion which has been strongly supported by subsequent x-ray diffraction studies. These latter studies showed that these lipids form unusually thin gel-state bilayers which do not exhibit the pronounced

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[1] *Abbreviations used:* DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; lyso-PC, 1-*O*-acyl lysophosphatidylcholine; 10:22 PC, 1-*O*-decanoyl, 2-*O*-docosanoyl phosphatidylcholine; 20:12 PC, 1-*O*-eicosanoyl, 2-*O*-dodecanoyl phosphatidylcholine; FTIR, Fourier transform infrared; T_m, gel/liquid-crystalline phase transition temperature; *R*, normalized splitting (i.e., the CH_2 (or CD_2) band splitting observed with the isotopically diluted sample divided by the band splitting observed with the fully proteated (or fully perdeuterated) sample (see Snyder et al., 1992)).

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central trough in the electron density typical of the localized methyl termini of symmetric chain lipids (McIntosh et al., 1984; Shah et al., 1990). Moreover, the mean area occupied by the lipid polar headgroup of these markedly chain asymmetric PCs is approximately three times that of an all-*trans* hydrocarbon chain instead of two times the hydrocarbon chain area as observed with the more symmetric chain PCs (Hui et al., 1984). It was thus proposed that these lipids form an unique type of "mixed interdigitated" gel phase consisting of arrays of repeating units, each of which is composed of two opposed PC molecules. These "dimers" have their long hydrocarbon chains extending completely across the hydrophobic core of the bilayer but with the methyl termini of their short chains opposed across the center of the bilayer (McIntosh et al., 1984).

Although many studies of PCs which form this so-called mixed interdigitated gel phase have been reported, most such work deals with the thermodynamic properties of their phase transitions (McIntosh et al., 1984; Hui et al., 1984; Huang and Mason, 1986; Xu et al., 1987; Xu and Huang, 1987; Mattai et al., 1987; Wong et al., 1989; Shah et al., 1990; Huang, 1990; Slater et al., 1992) or with their miscibility with other lipids (Xu et al., 1987; Lin and Huang, 1988; Ali et al., 1989; Sisk et al., 1990; Blutmann et al., 1991;, Slater et al., 1992; Sisk and Huang, 1992), and relatively few studies have focused on the details of the structure and dynamics of mixed interdigitated gel-state bilayers. With the exception of the x-ray diffraction studies referenced above, the only other structural studies of mixed interdigitated bilayers reported to date are a high pressure Fourier transform infrared (FTIR) spectroscopic characterization of the gel-phase packing (Wong et al., 1989) and a ^{31}P-NMR spectroscopic characterization of the motion of the phosphate groups (Xu et al., 1987). The FTIR spectroscopic studies indicate that the hydrocarbon chains tend to pack with perpendicular zigzag planes in the mixed interdigitated gel phase, and the ^{31}P-NMR spectroscopic studies indicate that motion of the phosphate headgroup of these lipids is axially symmetric in both the gel and liquid-crystalline state. We have recently begun a broadly based study aimed at characterizing the way(s) in which the acyl chain asymmetry of mixed chain PC bilayers can affect hydrocarbon chain packing and interfacial hydration in the gel and quasi-crystalline phases formed, as well as acyl chain conformation and dynamics in the liquid-crystalline state. Our approach involves the use of specifically isotopically labeled lipids as spectroscopic probes of the structural properties of different types of mixed chain phospholipids. This paper reports the results of a FTIR spectroscopic characterization of the mixed-interdigitated gel phases formed by markedly chain asymmetric PCs and utilizes specifically chain deuterated and ^{13}C=O-labeled lipid analogues as infrared spectroscopic probes of hydrocarbon chain packing and interfacial hydration.

MATERIALS AND METHODS

The PCs used in this study were synthesized by the acylation of appropriate lyso-PCs with an appropriate fatty acid anhydride using 4-pyrrolidino pyridine as a catalyst (for full experimental details see Lewis and McElhaney (1992)). These PCs were subsequently purified by methods previously used in this laboratory (Lewis and McElhaney, 1985). The unlabeled lyso-PCs were obtained commercially (Avanti Polar Lipids, Alabaster, AB), whereas the chain-perdeuterated and ^{13}C=O-labeled lyso-PCs were synthesized from their respective diacyl-PCs by previously published methods (Mason et al., 1980). The isotopically labeled diacyl-PCs used to synthesize the lyso-PCs were themselves synthesized from the appropriate labeled fatty acids and purified by previously published methods (Lewis and McElhaney, 1985). Decanoic acid (d_{19}), dodecanoic acid (d_{23}), eicosanoic acid (d_{39}), and the ^{13}C=O fatty acids were obtained from commercial sources (MSD Isotopes, Montreal, Québec), whereas docosanoic acid (d_{43}) was synthesized by deuterium exchange methods (Hsiao et al., 1974), purified by column chromatography, and recrystallized from hexane. For the FTIR spectroscopic experiments, 2–3 mg of the dried lipid sample were dispersed in 50 μl of D_2O by vigorously vortexing at temperatures well above the T_m of the lipid. This dispersion was then squeezed between the BaF_2 windows of a heatable liquid cell (equipped with a Teflon spacer) to from a 10-μm film. Once mounted in the sample holder of the instrument, the sample temperature could be controlled (between −20° and 90°C) by a external, computer-controlled circulating water bath. The infrared spectra were recorded with a Digilab FTS-40 infrared spectrometer (Digilab, Cambridge, MA) using the acquisition parameters previously described by Mantsch et al. (1985). The spectra obtained were analyzed using software supplied by Digilab Inc. and other computer programs obtained from the National Research Council of Canada. In cases where absorption bands were clearly definable in terms of a summation of component bands, Fourier self deconvolution was used to obtain accurate estimates of the peak frequencies of the component bands. Specifically, for the C=O stretching bands of the *sn*2-^{13}C=O-labeled lipids, Fourier self-deconvolution was performed with the Digilab DDS software package using band-narrowing factors of 1.8–2, and band width parameters of 14–18 cm^{-1} and 20 cm^{-1} in the gel and liquid-crystalline phases, respectively. Subsequently, curve-fitting procedures were used to obtain estimates of the widths and integrated areas of the component bands by reconstructing the contours of the original absorption band. This was achieved by a linear combination of the component bands identified by Fourier self-deconvolution with the aid of standard nonlinear least squares minimization procedures. Each band was simulated by a Gaussian-Lorentzian function for which best fit estimates of band shape was achieved with approximately 70% Gaussian contribution. Under the conditions of these experiments, band-narrowing factors up to 2.2 could be used during Fourier self-deconvolution without introducing significant distortions into the deconvolved spectra.

RESULTS

In this study 10:22 PC and 20:12 PC were used as representative asymmetric chain lipids which meet previously defined criteria for the formation of mixed interdigitated gel phases and which, respectively, have short and long acyl chains esterified to the *sn*1 position of the glycerol backbone. For these lipids their mixed interdigitated gel to liquid-crystalline phase transitions occur at 37.8° and 34.0°C, respectively. Fig. 1 shows the CH_2 stretching, C=O stretching and CH_2 deformation regions of the infrared spectra of the mixed interdigitated gel and liquid-crystalline phases of 20:12 PC. The spectroscopic features shown therein are typical of all of the chain asymmetric PCs forming mixed interdigitated gel phases which we have studied and are independent of whether long fatty acyl chains are esterified to the *sn*1 or *sn*2 positions of the glycerol backbone. As shown in Fig. 1, the gel/liquid-crystalline phase transition of these lipids is accompanied by changes in the all three regions of the infrared spectrum. For example, in the CH_2 stretching region, this phase transition is accompanied by changes in the

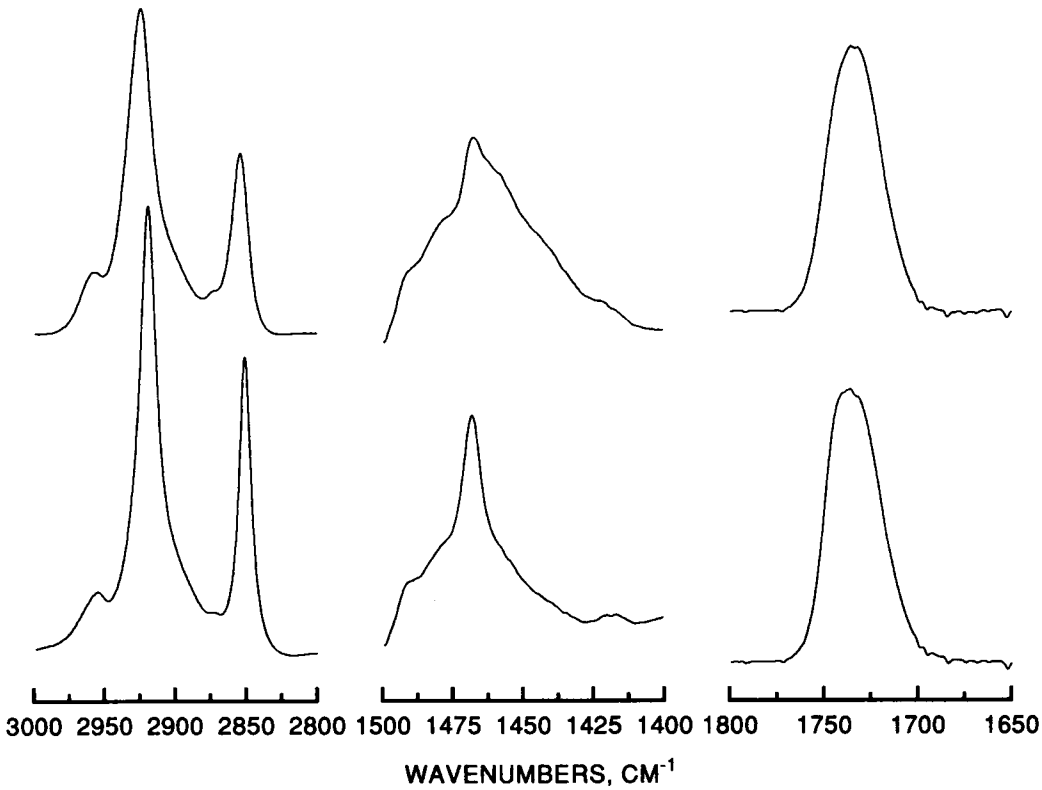

FIGURE 1 The CH_2 stretching (*left*), C=O stretching (*middle*), and CH_2 deformation (*right*) regions of the infrared spectrum of 20:12 PC. The band contours shown exemplify those observed with these types of asymmetric chain PCs and are representative spectra acquired at temperatures above (*top panel*) and just below (*bottom panel*) the gel/liquid-crystalline phase transition.

frequencies and widths of all bands present, whereas in the CH_2 deformation region of the spectrum, the gel/liquid-crystalline phase transition is accompanied by major changes in band width. Comparable changes have been observed at the gel/liquid-crystalline phase transitions of virtually all lipid bilayers studied so far and are diagnostic of the decrease in the conformational order (CH_2 stretching region) and the increases chain mobility (CH_2 deformation changes) which occur at the chain-melting phase transition. Since such changes have been extensively characterized in numerous studies of the gel/liquid-crystalline phase transition of many different types of lipid bilayers (for reviews see Mendelsohn and Mantsch (1986) and Mantsch and McElhaney (1991)), these results will not be discussed further.

Fig. 1 also shows the changes in the contours of the C=O stretching band which occur at the gel/liquid-crystalline phase transition of these lipids. In the liquid-crystalline phase, the C=O stretching band exhibits a broad contour centered near 1735 cm^{-1} which is essentially similar to that exhibited by most other hydrated PC bilayers. However, with the conversion to the mixed interdigitated gel phase, there is an increase in the intensity of infrared absorption near 1741 cm^{-1} relative to other regions of the C=O stretching band (see Fig. 1) and, as illustrated in Fig. 2 (*right panel*), this trend is accentuated as the temperature is decreased. Such changes in the contours of the C=O stretching band are probably a reflection of the fact that the observed C=O stretching band is a summation of underlying components, the properties of which are both temperature- and phase state-dependent (see Mendelsohn and Mantsch (1986) and references cited therein). In fully hydrated 1,2-diacylglycerolipid bilayers the C=O stretching band is known to consist of two components which are centered near 1743 and 1728 cm^{-1} (see Mendelsohn and Mantsch (1986) and references cited therein). It was originally proposed that these components arise from the stretching vibrations of *sn*1- and *sn*2-ester carbonyl groups, respectively, primarily because of their conformational differences (Levin et al., 1982; Mushayakarara et al., 1982; Mushayakarara and Levin, 1982 and references cited therein). However, recent studies of hydrated bilayers of specifically ^{13}C=O-labeled 1,2-diacylphospholipids have shown these components arise from a summation of comparable contributions of both the *sn*1- and *sn*2-ester carbonyl groups (Blume et al., 1988; Lewis and McElhaney, 1992). From such studies it became evident that the known conformational differences between the *sn*1- and *sn*2-ester carbonyl groups of those diacyl glycerolipids can only account for a 3–4 cm^{-1} difference between their peak frequencies instead of the 13–15 cm^{-1} which is experimentally observed. However, the experimentally determined frequency differences are within the range expected of hydration and hydrogen-bonding effects (Mushayakarara et al., 1986; Blume et al., 1988), and it is thus reasonable to assign those two components to the stretching vibrations of subpopulations of "free" and hydrogen-bonded ester carbonyl groups (see Blume et al., 1988). We thus suggest that

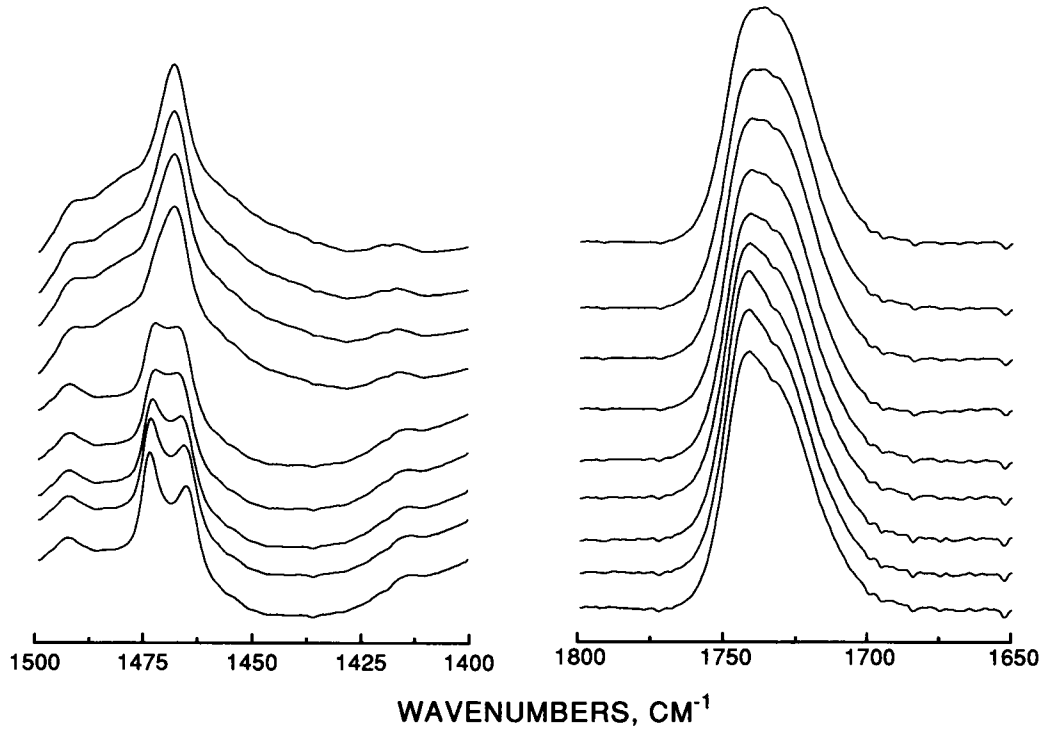

FIGURE 2 Temperature-dependent changes in the contours of the CH_2 scissoring (*left*) and C=O stretching bands (*right*) of 10:22 PC. The absorbance spectra shown were acquired at the temperatures ranging from −20°C (*bottom*) to 20°C (*top*) in increments of 5°C.

conversion to the mixed interdigitated gel phases results in marked changes in hydration and hydrogen-bonding interactions in the bilayer polar/apolar interface near to the ester C=O groups, changes which are not typically observed with "normal" symmetric chain PCs. However, since the C=O bands shown in Figs. 1 and 2 are probably a summation of contributions arising from subpopulations of free and hydrated (hydrogen-bonded) *sn*1- and *sn*2-ester carbonyl groups, a more detailed interpretation required that the contributions arising from the *sn*1- and *sn*2-ester carbonyl groups be specifically identified. This was achieved by the use of specifically ^{13}C=O-labeled analogues of these lipids.

Fig. 3 shows the C=O stretching regions of infrared spectra of unlabeled 20:12 PC (*left panel*) and the corresponding spectra obtained in parallel studies of *sn*2-^{13}C=O-labeled samples of that lipid (*right panel*). With the *sn*2-^{13}C=O-labeled lipid, the absorptions arising from the *sn*1-^{12}C=O and *sn*2-^{13}C=O-ester carbonyl groups of liquid-crystalline samples are centered near 1735 and 1692 cm^{-1}, respectively. Irrespective of the temperature or phase state of the sample, we find that the C=O stretching band contours of any unlabeled sample can be reconstructed by a simple addition of the absorption bands of the *sn*1-^{12}C=O and *sn*2-^{13}C=O-ester carbonyl groups of the corresponding specifically labeled sample, after a 42 cm^{-1} correction of the ^{13}C=O absorption band due to the "isotopic shift." The magnitude of the observed ^{12}C=O-^{13}C=O isotopic shift is similar to that reported in previous studies of hydrated phospholipid bilayers composed of specifically ^{13}C=O-labeled lipids (see Blume et al., 1988; Lewis and McElhaney, 1992). From an examination of the spectra shown in Fig. 3, it is also evident that the two bands are well resolved (especially at low temperatures) and that the contours of both the *sn*1 and *sn*2 absorption bands are phase state- and temperature-dependent. Also, from an examination of the spectra (especially the ^{12}C=O band), the changes in the contours of these bands seem to arise from changes in the relative contributions of underlying component bands. Using a combination of Fourier self-deconvolution and curve-fitting procedures, we find that in the liquid-crystalline state the *sn*1-ester C=O band itself seems to be a composite of two bands (see *dashed curves* in Fig. 3) which are of comparable integrated intensity and centered near 1742 and 1728 cm^{-1}, whereas the *sn*2-ester carbonyl band seems to consist of two components (centered at 1700 and 1687 cm^{-1}) of which the lower frequency component is of greater integrated intensity. Also, as illustrated in Fig. 3, the conversion to the mixed interdigitated gel phase seems to be accompanied by a marked increase in the relative intensity of the *sn*1-^{12}C=O band near 1742 cm^{-1} (presumably at the expense of the *sn*1-^{12}C=O band near 1728 cm^{-1}), and a marked decrease in the relative intensity of the *sn*2-^{13}C=O band near 1700 cm^{-1} coupled with relative increases in the intensity of the *sn*2-^{13}C=O band near 1687 cm^{-1}. As is also apparent from Fig. 3, these trends are more pronounced at lower temperature. Given the arguments presented in the preceding paragraph, it seems reasonable to assign the absorption bands near 1742 cm^{-1} (*sn*1-^{12}C=O) and 1700 cm^{-1} (*sn*2-^{13}C=O) to "free" *sn*1- and *sn*2-ester carbonyl groups, respectively, and the absorption bands near 1728 cm^{-1} (*sn*1-^{12}C=O) and 1687 cm^{-1} (*sn*2-^{13}C=O) to

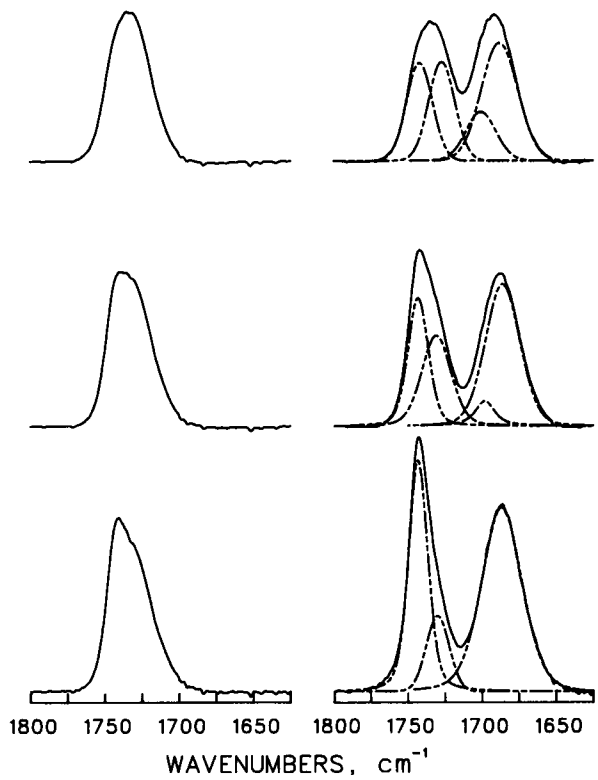

FIGURE 3 The C=O stretching region of the infrared spectrum of unlabeled (*left panel*) and *sn*2-^{13}C=O-labeled 20:12 PC (*right panel*). Spectra are presented for: the liquid-crystalline phase at 40°C (*top*); the gel phase at 20°C (*middle*); the gel phase at −20°C (*bottom*). Data are presented in the absorbance mode with the solid line representing the observed band contours and the dashed lines representing estimates of the component bands obtained by a combination of Fourier deconvolution and curve-fitting.

hydrogen-bonded (hydrated) ester carbonyl groups, respectively. Our results therefore suggest that the mixed interdigitated gel phases of these lipids are enriched in lipids and containing free *sn*1- and hydrogen-bonded *sn*2-ester carbonyl groups. This conclusion has interesting structural implications which will be explored further in the Discussion.

Another feature of the mixed interdigitated gel phases of these lipids is the evolution of a pronounced factor group splitting of the CH_2 scissoring band near 1470 cm^{-1} as the temperature is decreased. As illustrated in Fig. 2, the apparently single CH_2 scissoring band near 1468 cm^{-1} observed at temperatures just below the liquid-crystalline/gel phase transition, broadens significantly upon cooling and eventually splits into two narrower bands which are centered at 1466 and 1472 cm^{-1}. The splitting of the CH_2 scissoring (and the rocking) bands of PCs which form mixed interdigitated gel phases have been observed in previous high-pressure FTIR spectroscopic studies (Wong and Huang, 1989). This observation suggests that in the mixed interdigitated gel phase, the hydrocarbon chains of these lipids tend to pack with their zigzag planes perpendicular to each other (probably in an orthorhombic $\perp$ or $\perp'$ subcell) once chain reorientational fluctuations are damped by either low temperature (this work) or high pressure (Wong and Huang, 1989). Given that the "structural repeat unit" of the mixed interdigitated gel phases of these lipids is believed to be a "triple chain" assembly consisting of two PC molecules in which their long chains project across the entire bilayer with the methyl termini of their short chains meeting at the center of the bilayer (McIntosh et al., 1984), these spectroscopic data raise some interesting questions about the assembly of these structural units into a lattice of orthorhombic $\perp$ (or $\perp'$) subcells. By definition, the assembly of these "structural repeat units" into such a lattice can only be accomplished if the number of sites occupied by long chains is twice that occupied by short chains. This condition, when combined with conditions intrinsic to both the type of subcellular packing and the structure of the individual lipid molecules (e.g., perpendicular packing of the hydrocarbon chains, and the fact that each long chain is physically linked to a short chain), imposes severe restrictions on the types of packing formats that are feasible. Thus, we find that the condition that there be a global motif of perpendicularly packed hydrocarbon chains and the requirement that the sites occupied by long chains be twice as numerous as those occupied by short chains can only be simultaneously satisfied by a packing motif that is essentially similar to that illustrated in Fig. 4 (or some variation thereof). The critical feature of the packing format illustrated in Fig. 4 is that each short chain is surrounded by four perpendicularly aligned long chains, whereas each long chain is surrounded by two perpendicularly aligned long chains and two perpendicularly aligned short chains. Consequently, once packed in this manner lateral close contact interactions involving short chains will occur exclusively with long chains, whereas each long chain will have lateral close contact interactions with both long and short chains. This is important because the type of band splitting described above generally arises when close contact interactions between all-*trans*, orientationally inequivalent polymethylene chains result in interchain coupling of their CH_2 scissoring vibrations (Snyder, 1961, 1979). These considerations made it feasible to test the validity of the packing motif suggested in Fig. 4 by a judicious use of specifically chain-perdeuterated analogues of these lipids.

Illustrated in Fig. 5 are the CH_2 deformation regions of the infrared spectra of unlabeled, *sn*1 chain-perdeuterated, and *sn*2 chain-perdeuterated samples of 20:12 PC (1-*O*-long-chain, 2-*O*-short-chain) and 10:22 PC (1-*O*-short-chain, 2-*O*-long-chain). An interesting feature of the data shown is the fact that irrespective of the length of the fatty acyl chains, a relatively weak absorption band near 1418 cm^{-1} is present in the spectra of the unlabeled and *sn*2 chain-perdeuterated samples, but absent from the spectra of the *sn*1 chain-perdeuterated samples. This band has been assigned to the scissoring vibrations of the α-methylene groups of fatty acyl chains (see Mendelsohn and Mantsch (1986)). Thus its absence from the spectra of the *sn*1 chain-perdeuterated sample indicates that in these hydrated lipid bilayers the infrared absorption near 1418 cm^{-1} arises predominantly from the scissoring vibrations of α-methylene groups on *sn*1 fatty acyl chains. Similar results have been reported in infrared spectroscopic studies of the subgel phases of symmetric chain

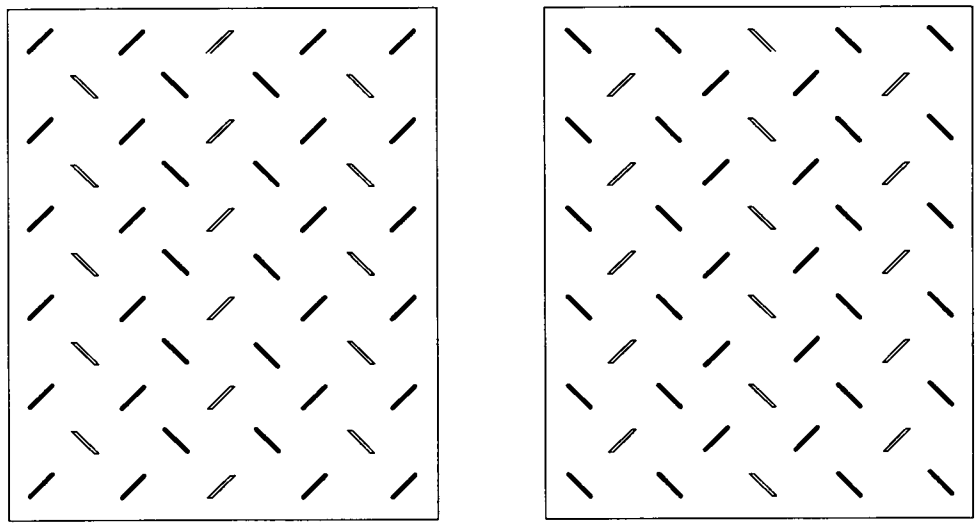

FIGURE 4 Diagram illustrating the arrangement of long and short chains in a bulk subcellular lattice in which the hydrocarbon chains are perpendicular to each other and with long chains occupying twice as many lattice sites as do short chains. As drawn the *ab* plane of the lattice is in the plane of the page, with the lined symbols indicating the orientation of the zigzag planes of the hydrocarbon chains. The filled symbols represent the sites occupied by long chains and the open symbols represent the sites occupied by short chains.

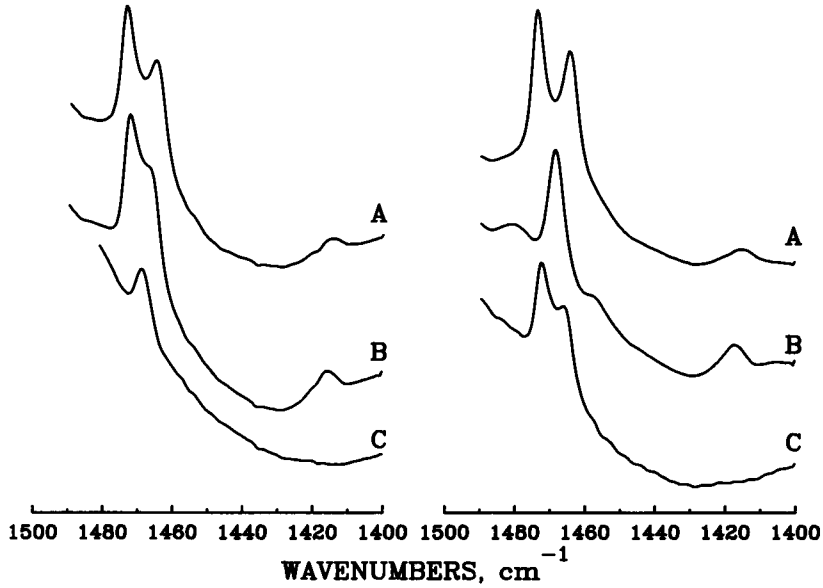

FIGURE 5 The CH_2 bending region of 20:12 PC (*left panel*) and 10:22 PC (*right panel*). The absorbance spectra shown were acquired at −20°C for aqueous dispersions of: (*A*) Unlabeled samples; (*B*) *sn*2 chain-perdeuterated samples; (*C*) *sn*1 chain-perdeuterated samples.

n-saturated diacyl-PCs (Lewis and McElhaney, 1992). Currently, it is unclear why the *sn*2 α-methylene groups do not contribute significantly to infrared absorption in this part of the spectrum. However, it has been suggested that this may be related to conformational differences between the *sn*1 and *sn*2 α-methylene groups (Lewis and McElhaney, 1992). If such is the case, then the above observations also suggest that the conformational inequivalence between α-methylene groups of the *sn*1 and *sn*2 acyl chains observed in normal symmetric chain phospholipids (see Hitchcock et al., 1974; Pearson and Pascher, 1979; Hauser et al., 1981, 1988) is preserved in the mixed interdigitated gel phases of these lipids.

The spectra presented in Fig. 5 also indicate that the CH_2 scissoring bands of the unlabeled samples of both lipids are strongly split into components centered at 1466 and 1472 cm^{-1}. With those samples in which the short chains have been perdeuterated, the observable splitting is attenuated to approximately 70% that observed in the unlabeled sample, irrespective of whether the short chains are esterified to the *sn*1 (R = 0.74 at −20°C) or *sn*2 (R = 0.67 at −20°C) position of the glycerol backbone (see spectra of *sn*2 chain-perdeuterated 20:12 PC and *sn*1 chain-perdeuterated 10:22 PC). The attenuation of the splitting is consistent with a reduction in size of the domains of perpendicularly packed, laterally interacting fully proteated polymethylene chains (see Bank and Krimm, 1969; Snyder et al., 1992) and indicates that perdeuteration of the short chains uncouples some but not all of the lateral close contact vibrational interactions involving the long chains of these lipids. However, for those

analogues in which the long chains have been perdeuterated, a single CH_2 scissoring band centered at 1468 cm^{-1} is observed (see spectra of *sn*1 chain-perdeuterated 20:12 PC and *sn*2 chain-perdeuterated 10:22 PC). In this case the CH_2 scissoring band appears as a single sharp band for which there is no evidence of residual band splitting, even if band narrowing techniques such as Fourier self-deconvolution are employed. Thus perdeuteration of the long fatty acyl chains of these lipids uncouples all lateral close contact vibrational interactions involving the short fatty acyl chains, thereby causing a complete collapse of the CH_2 scissoring band splitting. It should be noted, however, that the above observations are not simply the result of a 2:1 (^2H:^1H) dilution of the proteated hydrocarbon chains in the lattice. In previous isotope dilution studies using long chain hydrocarbons, it was demonstrated that there is obvious CH_2 scissoring band splitting with presumably random distributions of ^2H- and ^1H-labeled hydrocarbon chains in an orthorhombic lattice, even when 70 mol% of the hydrocarbon chains are perdeuterated (see Bank and Krimm, 1969; Spells et al., 1980; Snyder et al., 1992). Those studies showed that the CH_2 scissoring band splitting is clearly resolvable as a doublet only when the concentration of the proteated chains exceeds 50 mol%. However, at lower ^1H concentrations there still is a residual CH_2 scissoring band splitting which is manifest by an increase in band width (Spells et al., 1980) and by a distortion of the band shape to form distinct shoulders mainly toward the higher frequency range of the absorption band (Bank and Krimm, 1969; Snyder et al., 1992). In our experiment, the observed complete collapse of the CH_2 scissoring band splitting at the levels of chain perdeuteration achieved by the perdeuteration of the long chains of these lipids is atypical of comparable levels of random isotopic dilution but is precisely what would be predicted if the hydrocarbon chains of these lipids adopt a packing motif comparable to that illustrated in Fig. 4. From the chain packing motif proposed, it is evident that the short chains in the lattice will interact exclusively with long chains. Thus, since perdeuteration changes the natural frequency of the methylene scissoring vibrations (from $\cong$1470 cm^{-1} for CH_2 to $\cong$1088 cm^{-1} for CD_2), perdeuteration of the long chains should completely uncouple the CH_2 scissoring vibrations of the methylene groups on the short chains and no band splitting should occur. However, since each long chain in the lattice will interact with both short and long chains, perdeuteration of the short chains will not affect any vibrational coupling arising from interactions between neighboring long chains, but it will uncouple the vibrational interactions between long chains and short chains. Thus when the short chains are perdeuterated, band splitting should be attenuated but not obliterated as does occur when the long chains are perdeuterated. These results thus provide strong evidence that the mixed interdigitated gel phase is composed of the triple chain structural repeat units proposed earlier and that their hydrocarbon chains do adopt a packing motif comparable to that proposed above. This conclusion has important implications for the assembly of triple chain repeat units in the mixed interdigitated gel phase (see below).

DISCUSSION

A seemingly minor finding of this study is that whether viewed from the perspective of the hydrocarbon chain packing motif or the organization of the C=O-ester polar/apolar interface, mixed interdigitated gel phases composed of 1-*O*-long chain, 2-*O*-short chain PCs are spectroscopically indistinguishable from those composed of 1-*O*-short chain, 2-*O*-long chain PCs. Although peripheral to the main thrust of this work, this observation is significant, because it suggests that in the mixed interdigitated gel phase, the global aspects of hydrocarbon chain packing, and interfacial hydration and conformation, are independent of whether the longer acyl chain is esterified at the *sn*1 or *sn*2 position of the glycerol backbone. Moreover, the above observation also suggests that in their respective mixed interdigitated gel phases, the molecular conformation adopted by individual 1-*O*-short chain, 2-*O*-long chain PCs is similar to that adopted by their 1-*O*-long chain, 2-*O*-short chain counterparts. The above observations are significant, because they simplify further structural interpretation of our data. Specifically, they effectively eliminate the need for considering the possibility that molecular conformation depends upon the position at which the long or short chains are esterified.

The FTIR spectroscopic studies of the unlabeled and specifically ^{13}C=O-labeled samples of these lipids also indicate that for lipids which form mixed interdigitated gel phases, the gel/liquid-crystalline phase transition is accompanied by larger structural changes at their polar/apolar interfaces than that which occurs at the hydrocarbon chain-melting phase transitions of most normal symmetric chain PCs. Such structural changes evidently affect the contours of the stretching bands of the *sn*1- and *sn*2-ester carbonyl groups, and are primarily reflected by changes in the relative intensities of the subcomponents of both the *sn*1 and *sn*2-absorption bands. Within the context of hydrated lipid bilayers, opinion is still divided as to whether the existence of such subcomponents is primarily a reflection of conformational differences between *sn*1- and *sn*2-ester carbonyl groups (see Levin et al., 1982; Mushayakarara et al., 1982; Mushayakarara and Levin, 1982, and references cited therein), or of instantaneous populations of free and hydrogen-bonded ester carbonyl groups (see Blume et al., 1988; Lewis and McElhaney, 1992 and references cited therein). However, the studies of specifically ^{13}C=O-labeled lipids have shown that conformational differences between the *sn*1- and *sn*2-ester C=O groups of hydrated PC bilayers cannot account for the magnitude of the differences in the peak frequencies of the subcomponents of the C=O stretching bands of these lipids, and as demonstrated previously, differences in the populations of free and hydrogen bonding ester C=O groups can easily account for such differences (see Mushayakarara et al., 1986;

Blume et al., 1988). Consequently, we have assigned the components of the C=O stretching band to subpopulations of free and hydrogen-bonded ester carbonyl groups and have interpreted our data accordingly. Given this we therefore propose that normal symmetric chain PCs and the highly asymmetric mixed chain PCs described here differ significantly with respect to the degrees of interfacial hydration and hydrogen bonding of their respective ester carbonyl groups. From previous studies of specifically ^{13}C=O-labeled symmetric chain PCs, one can conclude that the populations of free and hydrogen-bonded *sn*1- and *sn*2-ester carbonyl groups undergo relatively minor changes at the gel/liquid-crystalline phase transition, and that major redistributions of these populations only occur when these lipids form their respective quasicrystalline subgel (L_c) phases (Blume et al., 1988; Lewis and McElhaney, 1992). However, with the particular class of asymmetric chain PCs studied here, the formation of their mixed interdigitated gel phases is accompanied by a major redistribution of the populations of free and hydrogen bonded ester carbonyls at both the *sn*1 and *sn*2 positions. Specifically, the formation of the mixed interdigitated gel phase is accompanied by major increases in the populations of free *sn*1 and hydrogen-bonded *sn*2 carbonyl groups, at the obvious expense of the respective populations of hydrogen-bonded *sn*1 and free *sn*2 groups. Since interfacial water is the primary source of hydrogen-bond donors to which the carbonyl groups could be bonded, the observed spectroscopic changes suggest that as the mixed interdigitated gel phase is formed and as that phase becomes more compact when the temperature is decreased, the *sn*1-ester carbonyls become more shielded from available pools of interfacial water, whereas the *sn*2 groups become less shielded from those sources of hydrogen-bonding donor groups. These conclusions are consistent with the structural model of the mixed interdigitated gel phase as deduced from x-ray diffraction studies (see McIntosh et al., 1984). In this model, the long chains of these lipids are fully interdigitated across the bilayer with the methyl termini of the short chains opposed across the center of the bilayer. Since such an arrangement requires that the methyl termini of long acyl chains of any given monolayer be located near to the carbonyl groups of *sn*1 chains of the opposing monolayer, the regions near to the *sn*1-ester carbonyl groups may become more hydrophobic and, as a consequence, less amenable to penetration by interfacial water. The exclusion of water from (or its restricted access to) this region of the bilayer polar/apolar interface should thus be conducive to the increases in the population of free *sn*1-ester carbonyls that was deduced from our experimental results. We can also suggest that the above processes would be promoted as hydrocarbon chain packing becomes tighter at lower temperatures.

The existence of relatively large populations of hydrogen-bonded *sn*2-ester carbonyl groups in the mixed interdigitated gel phases of these lipids can also be rationalized within the context of the structural model proposed. Implicit in this model is the idea that the polar surface of the structural repeat consists of three acyl chains per polar headgroup, a suggestion which is supported experimentally (Hui et al., 1984). Assuming that the usual conformational differences between the *sn*1 and *sn*2 acyl chains are maintained with these asymmetric chain lipids (see below), one would expect that the *sn*2 carbonyl groups of both normal symmetric chain lipids and these particular asymmetric chain PCs should project into fairly polar regions just below the plane of the headgroups. With normal symmetric chain PCs, the density of polar headgroups per unit bilayer surface area is greater than that which exists in mixed interdigitated bilayers, and we therefore propose that with the former, larger populations of free (i.e., nonhydrogen bonded) *sn*2-ester carbonyls can exist because the relatively high surface density of polar headgroups can effect some steric shielding of the *sn*2 carbonyl groups from potentially large pools of bulk and interfacial water. With these particular asymmetric chain PCs, such shielding would be considerably attenuated because of the lower surface density of polar headgroups, and this should permit the formation of relatively larger populations of hydrogen-bonded ester carbonyl groups than is observed with their symmetric chain counterparts. We also suggest that this process could be further enhanced at lower temperatures, because the shielding effects of the polar headgroups would be further attenuated as their mobility is reduced.

Interestingly, our results suggest that the liquid-crystalline phases of this class of lipids may also differ from those of normal symmetric chain PCs as regards the relative populations of free and hydrogen-bonded ester carbonyl groups present at their respective polar/apolar interfaces. With normal symmetric chain PCs, FTIR spectroscopic studies of specifically ^{13}C=O-labeled analogues indicate that the absorption bands of *sn*1- and *sn*2-ester C=O stretching vibrations are each the summation of two components, which have been assigned to subpopulations of free and hydrogen-bonded carbonyl groups (see Blume et al., 1988). With such lipids, the integrated intensities of these bands tend to be comparable in the liquid-crystalline state (Blume et al., 1988). With these asymmetric chain lipids, however, our analyses indicate that even in the liquid-crystalline phase the infrared absorption ascribable to hydrogen-bonded *sn*2-ester carbonyl groups greatly exceeds that of the free *sn*2 carbonyl groups (see Fig. 3). Since it seems unlikely that the liquid-crystalline phases of these lipids would be more hydrated at their polar/apolar interfaces than are those of normal symmetric chain PCs, the above result may simply be a reflection of greater exposure of the *sn*2 carbonyl groups to the pool of available hydrogen-bonding donors, possibly because of less effective steric shielding by the polar headgroups.

Our studies of the unlabeled and chain-perdeuterated lipids provide very detailed information about hydrocarbon chain packing in the mixed interdigitated gel phase and this data makes possible fairly detailed proposals about the ways in which individual PC molecules are assembled in this phase. An interesting though peripheral finding of this study is that the absorption band observed near 1420 cm^{-1} arises

predominantly (or even exclusively) from the scissoring vibrations of α-methylene groups on *sn*1-esterified fatty acyl chains. Similar results were obtained in studies of the subgel phases of *n*-saturated symmetric chain PCs and ascribed to conformational differences between the α-methylene segments of the *sn*1 and *sn*2 fatty acyl chains (Lewis and McElhaney, 1992). Our results therefore suggest that there are also comparable conformational inequivalences between the α-methylene segments of the *sn*1 and *sn*2 chains of these asymmetric chain PCs. That our data supports such a conclusion is not unexpected, since such conformational inequivalence is implicitly assumed in all models proposed to rationalize structural and thermodynamic properties of symmetric chain PCs and indeed the majority of mixed chain PCs studied so far (Mason and Huang, 1981; Davis and Keough, 1985; Huang, 1991; Marsh, 1992).

An important aspect of our studies of the chain perdeuterated lipids is the strong support for the previously proposed structure in which the mixed interdigitated gel phase is an assembly of triple chain repeat units with perpendicularly packed hydrocarbon chains in a packing motif comparable to that proposed in Fig. 4. Our evidence that 1-*O*-long chain, 2-*O*-short chain PCs adopt similar conformations as do their 1-*O*-short chain, 2-*O*-long chain counterparts makes feasible more detailed considerations of the way(s) in which the repeat units can be assembled into the proposed chain-packing motif and how pairs of individual PC molecules can be combined to form the repeat units. Illustrated in Fig. 6 are diagrams outlining the four simplest ways in which this can be achieved. We find that the four packing formats illustrated, and hybrids thereof, are the only ways in which the proposed triple chain repeat units can be assembled while retaining consistency with the requirements of the overall hydrocarbon chain packing motif and the other structural limitations described earlier. One should note that compatible lattices can be constructed with repeat units in which the long and short chains of each repeat unit are perpendicular to each other (Fig. 6, *A–C*), as well as repeat units in which the hydrocarbon chains of each repeat unit are parallel to each other (Fig. 6 *D*).

On the basis of previous high-pressure FTIR spectroscopic studies (Wong and Huang, 1989), a packing model was proposed in which the long and short chains (i.e., the *sn*1 and *sn*2 chains) forming each repeat unit were perpendicular to each other (see Huang (1990) and references cited therein). The assumption that the long and short chains of each repeat unit are perpendicular to each other has some very interesting implications. For example, if one assumes that, in the gel state, the lipid molecules adopt the generally accepted conformation in which the zigzag plane of the all-*trans sn*1 fatty acyl chain is continuous with that of the C1 and C2 of the glycerol backbone (see Hauser et al. (1981, 1988); Pearson and Pascher (1979); and Hitchcock et al. (1974)), then the requirement that the zigzag planes of *sn*1 and *sn*2 chains be perpendicular to each other will ensure that the orientation of the *sn*1 and *sn*2 chains relative to the zigzag of the glycerol backbone will be different, and as a result the shape of repeat

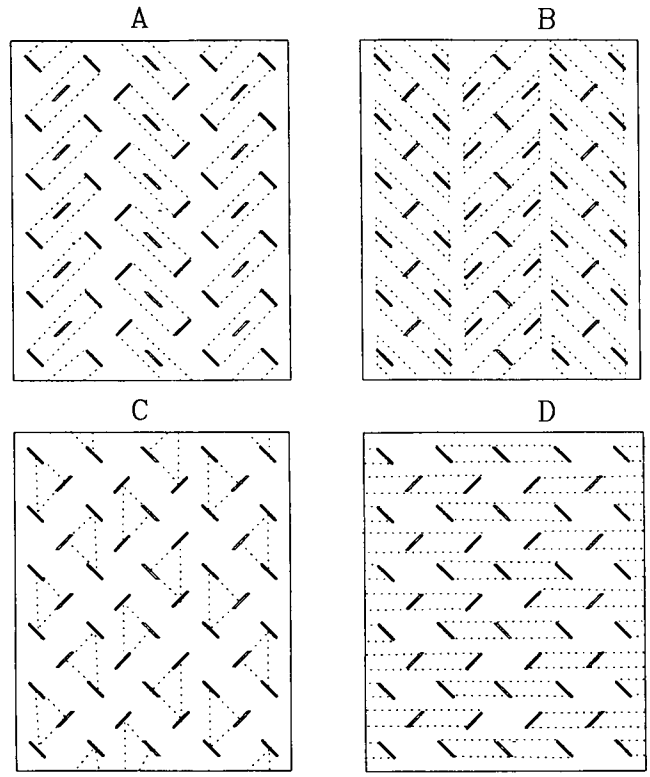

FIGURE 6 Diagram illustrating possible assemblies of triple chain structural repeat units in the mixed interdigitated gel phases of the asymmetric chain PCs. The lined symbols and hydrocarbon chain packing motif are the same as illustrated in Fig. 4, and the dashed lines connect the three lattice sites which are occupied by the hydrocarbon chains of PCs forming the triple chain repeat units. The diagrams represent possible arrangement of the repeat units in assemblies in which the hydrocarbon chains of the repeat unit are either perpendicular (*A–C*) of parallel (*D*).

units consisting of 1-*O*-long chain, 2-*O*-short chain lipids will differ from those consisting of 1-*O*-short chain, 2-*O*-long chain lipids. The differences in the shape of the repeat units will, in turn, require that there be differences in the way in which the repeat units are assembled in the lattice. Thus, if overall similarities of the molecular conformations adopted by 1-*O*-long chain, 2-*O*-short chain and 1-*O*-short chain, 2-*O*-long chain molecules are assumed and if the repeat units composed of 1-*O*-short chain, 2-*O*-long chain lipids are assembled as illustrated in Fig. 6 *A*, then the repeat units composed of the 1-*O*-long chain, 2-*O*-short chain lipids must be assembled as illustrated in Fig. 6 *B*. Also, given such assumptions, there are interesting implications pertinent to the repeat units which can be formed in a mixed interdigitated gel phase composed of a binary mixture of a 1-*O*-long chain, 2-*O*-short chain lipid with a comparable 1-*O*-short chain, 2-*O*-long chain species. In principle, such a system should consist of a mixture of triple chain repeat units composed of two identical PC molecules (i.e., the two homodimeric triple chain repeat units as described above), and heterodimeric repeat units composed of one 1-*O*-short chain, 2-*O*-long chain lipid opposed to a 1-*O*-long chain, 2-*O*-short chain species. With the latter, the methyl end of the *sn*1 short chain of one lipid will be opposed against the methyl terminus of

the *sn*2 short chain of the other lipid species, and the *sn*1 long chains of one species will be interdigitated with *sn*2 long chains of the other lipid. In our attempts at assembling such heterodimeric repeat units, we find that, because of the requirement for perpendicular packing of the *sn*1 and *sn*2 acyl chains and the resulting orientational inequivalence between the *sn*1 and *sn*2 chains relative to the zigzag of the glycerol backbone, the repeat units can only be assembled in the manner illustrated in Fig. 6 *C*. Such an assembly is evidently distinct from the two possible homodimeric types described above.

As demonstrated above, the precondition of perpendicular packing of the hydrocarbon chains within each repeat unit of the mixed interdigitated gel phase would result in the formation of differently shaped repeat units, depending on whether long or short chains are esterified to the *sn*1 position of the glycerol backbone, and on whether the repeat unit is "homodimeric" or "heterodimeric" as defined above. This finding has important implications for the gel phase miscibility of binary mixtures composed of a 1-*O*-long chain, 2-*O*-short chain and a 1-*O*-short chain, 2-*O*-long chain species. A previous study has shown that binary mixtures composed of a 1-*O*-long chain, 2-*O*-short chain PC and a 1-*O*-short chain, 2-*O*-long chain species for which the effective lengths[2] of the respective short and long chains of the two lipid species are comparable, exhibit near ideally in all proportions in both the gel and liquid-crystalline states, and that they also form the mixed interdigitated gel phase (Ali et al., 1989; Xu et al., 1987). Given such data, it is evident that if there is perpendicular packing of the hydrocarbon chains forming each triple chain repeat unit of the mixed interdigitated gel phase, then the latter would have to be a close packed assembly of three differently shaped repeat units. With such a system, however, there will be problems in accommodating the specific packing requirements of each of the three repeat units while ensuring compatibility with the overall packing motif. Although the latter could be achieved by the hybridization of the packing formats *A*, *B*, and *C* shown in Fig. 6, we find that it does require the formation of local extended arrays of the three possible types of repeat units (two homodimeric and one heterodimeric). We therefore suggest that, since the formation of such local extended arrays of differently shaped repeat units is entropically unfavorable, and is also incompatible with the near ideal gel phase mixing which has been observed experimentally (Ali et al., 1989; Xu et al., 1987), the long and short acyl chains of each structural repeat units may not be perpendicularly packed *within those units* as previously assumed (Huang, 1990).

Interestingly, the various packing problems described above do not emerge when one examines the possibility that the hydrocarbon chains within each repeat unit are arranged parallel to each other. Despite parallel arrangements of the acyl chains within each repeat unit, the global motif of perpendicularly packed acyl chains can be attained by the perpendicular stacking of the repeat units themselves (see Fig. 6 *D*). With such a packing format, the orientation of the zigzag planes of *sn*1 and *sn*2 fatty acyl chains to the zigzag of the glycerol backbone would be equivalent and, as a result, repeat units consisting of 1-*O*-long chain, 2-*O*-short-chain homodimers would have the same general shape (in terms of the hydrocarbon chain packing requirements) as would repeat units consisting of 1-*O*-short chain, 2-*O*-long chain homodimers. Moreover, in binary mixtures composed of 1-*O*-long chain, 2-*O*-short chain lipids and comparable 1-*O*-short chain, 2-*O*-long chain species, heterodimeric repeat units with the same shape and hydrocarbon chain packing requirements as those of the two possible homodimeric repeat units can be formed, provided that the effective lengths of the respective short and long chains of the two lipid species are comparable. In this packing format, the sites occupied by each triple chain repeat unit (and indeed each phospholipid molecule) are equivalent, and can be occupied by either a 1-*O*-long chain, 2-*O*-short chain species or its 1-*O*-short chain, 2-*O*-long chain counterpart, provided that effective lengths of their hydrocarbon chains are comparable. Indeed, the proposed equivalence of all available sites is consistent with the near ideal gel phase mixing which has been observed experimentally (Ali et al., 1989; Xu et al., 1987). We therefore conclude that a packing format in which the hydrocarbon chains of the repeat unit are parallel is more entropically favorable, and is also more consistent with currently available data, than is any format in which the hydrocarbon chains of the repeat units are perpendicular.

Finally, our results, when combined with previously published work, enable the construction of fairly detailed picture of the mixed interdigitated gel phase formed by this particular class of PCs. The evidence supports a picture in which the mixed interdigitated gel phase remains as an assembly of triple chain repeat units at all temperatures at which it is stable. At temperatures well below T_m, this assembly is very compact with strong lateral interactions between the hydrocarbon chains (note the strong factor group splitting of the CH_2 scissoring bands at those temperatures). In this form the hydrocarbon chains of each triple chain repeat unit are parallel to each other and the repeat units themselves are stacked perpendicular to each other, such that the global hydrocarbon chain packing motif is an array of perpendicularly packed, all-*trans* polymethylene chains in which the sites occupied by long chains are twice as numerous as those occupied by short chains. At those temperatures the region of the bilayer polar/apolar interface around the *sn*1-ester carbonyl groups becomes less accessible to interfacial water, mainly because of the compactness of the packing, and the presence of the methyl termini of the long chains in that region. With an increase in temperature, the increased frequency and amplitude of thermal motions result in the formation of a more loosely packed structure in which increases in the reorientational fluctuations of the hydrocarbon chains weaken and eventually collapse the interchain vibrational coupling of the CH_2 scissoring and rocking vibrations. With the increase in

[2] The residual length after correction for the inequivalence between chains esterified at the sn1 and sn2 positions of the glycerol backbone.

frequency and amplitude of thermal motions, there are also increases in the mobility of the phosphate headgroup (Xu et al., 1987) and a greater penetration of water into the interfacial region near to the *sn*1-ester carbonyl group (note the increase in the intensity of infrared absorption attributable to hydrogen-bonded *sn*1-ester carbonyl groups). Further resolution and refinement of this picture are the focus of continuing study.

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