The Interfacial Structure of Phospholipid Bilayers: Differential Scanning Calorimetry and Fourier Transform Infrared Spectroscopic Studies of 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphorylcholine and Its Dialkyl and Acyl-Alkyl Analogs

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ABSTRACT The thermotropic phase behavior of aqueous dispersions of dipalmitoylphosphatidylcholine (DPPC) and its 1,2-dialkyl, 1-acyl 2-alkyl and 1-alkyl 2-acyl analogs was examined by differential scanning calorimetry, and the organization of these molecules in those hydrated bilayers was studied by Fourier transform infrared spectroscopy. The calorimetric data indicate that substitution of either or both of the acyl chains of DPPC with the corresponding ether-linked hydrocarbon chain results in relatively small increases in the temperature (<4°C) and enthalpy (<1 kcal/mol) of the lipid chain-melting phase transition. The spectroscopic data reveal that replacement of one or both of the ester-linked hydrocarbon chains of DPPC with its ether-linked analog causes structural changes in the bilayer assembly, which result in an increase in the polarity of the local environments of the phosphate headgroups and of the ester carbonyl groups at the bilayer polar/apolar interface. The latter observation is unexpected, given that ester linkages are considered to be intrinsically more polar that ether linkages. This finding cannot be satisfactorily rationalized unless the conformation of the glycerol backbones of the analogs containing ether-linked hydrocarbon chains differs significantly from that of diacyl glycerolipids such as DPPC. A comparison of the α-methylene scissoring bands and the methylene wagging band progressions of these lipids with the corresponding absorption bands of specifically chain-perdeuterated analogs of DPPC also supports the conclusion that replacement of the ester-linked hydrocarbon chains of DPPC with the corresponding ether-linked analog induces conformational changes in the lipid glycerol backbone. The suggestion that the conformation of glycerol backbones in the alkyl-acyl and dialkyl derivatives of DPPC differs from that of the naturally occurring 1,2-diacyl glycerolipid suggests that mono- and di-alkyl glycerolipids may not be good models of their diacyl analogs. These results, and previously published evidence that DPPC analogs with ether-linked hydrocarbon chains spontaneously form chain-interdigitated gel phases at low temperatures, clearly indicate that the properties of lipid bilayers can be substantially altered by small changes in the chemical structures of their polar/polar interfaces, and highlight the critical role of the interfacial region as a determinant of the structure and organization of lipid assemblies.

INTRODUCTION

The chemical structure of the glycerol backbone region of phospho- and glycoglycerolipid molecules in general, and in particular the nature of the chemical linkage between their glycerol backbones and the hydrocarbon chain, are known to affect the conformation and physical properties of such molecules, and thus the nature and thermodynamic stabilities of the supramolecular aggregates that they form in aqueous dispersion. For example, replacement of the ester-linked hydrocarbon chains of glycerolipids with the corresponding ether-linked chains usually results in modest increases in the hydrocarbon chain-melting phase transition temperature (T_m) and enthalpy change (ΔH) of the gel/liquid-crystalline phase transition (see Lewis and McElhaney, 1992a, and references cited therein). Moreover, the liquid-crystalline lamellar to reversed cubic or hexagonal phase transition temperatures of dialkyl phosphatidylethanolamines (PEs) or monoglycosyl diacylglycerols (MGDGs) are markedly reduced with respect to those of the corresponding diacyl analogs (see Lewis et al., 1996, and references cited therein). In addition, substitution of one or both of the ester-linked hydrocarbon chains of 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) with their ether-linked counterparts greatly enhances the capacity of this lipid to form hydrocarbon chain-interdigitated gel phases, although the formation of interdigitated gel phases is not observed in the corresponding dialkyl PEs and MGDGs (Kim et al., 1987a,b; Ruocco et al., 1985; Seddon et al., 1984; Kuttenreich et al., 1988; Sen et al., 1990; Hing et al., 1991; Hinz et al., 1991).

Other approaches to the study of the effects of lipid interfacial structure on lipid phase behavior have involved comparisons of glycerolipids with ester-linked hydrocarbon chains with appropriate amido-linked counterparts (Curatolo et al., 1982, 1985; Chowdhry et al., 1984b), comparisons of normal glycerolipids with analogs containing "conformationally restricted backbones" (Blume and Eibl, 1981; Singer et al., 1983), and comparisons of glycerolipids with 1,3-linked hydrocarbon chains with comparable 1,2-linked

Received for publication 3 October 1995 and in final form 1 March 1996.
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0006-3495/96/06/2736/11 $2.00

analogs (Eibl and Blume, 1979; Serralach et al., 1983; Stumpel et al., 1981; Chowdhry et al., 1984a; Dluhy et al., 1985). It has also been demonstrated that the propensity of some glycerolipids to form inverted nonlamellar phases can be altered by the chirality of the glycerol backbone (Mannock et al., 1992, 1994) and by the location of modestly sized hydrophobic groups near the glycerol backbone of the lipid (Lewis et al., 1994a). Such studies clearly show that lipid phase behavior and organization can be significantly altered by chemical changes in this portion of the lipid molecule. Such findings are not surprising, because this region of the glycerolipid molecule defines the interface between the polar headgroup and apolar hydrocarbon chains of all lipid phases formed in water, and alterations to the structure of this region will thus affect the strengths of lipid/lipid and lipid/water interactions in all hydrated lipid aggregates. Currently available data also suggest that the degree of hydration and nature of hydrogen bonding of water to moieties located at or near lipid polar/apolar interfaces can strongly influence the properties of lipid bilayers and other supramolecular assemblies.

The molecular basis for the marked sensitivity of lipid physical properties to the chemical structure of the polar/apolar interfacial regions of lipid aggregates in general, and of lipid bilayers in particular, is incompletely understood at present. In part, this can be attributed to the current shortage of relevant data that directly address fundamental principles underlying the influence of lipid interfacial structure on lipid phase behavior or the mechanisms through which this influence can be exerted. To address such issues, we have recently begun a broadly based study of how lipid phase behavior can be affected by the chemical structure and conformation of moieties located at the polar/apolar interfaces of lipid bilayers. We present here the results of our initial differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopic studies of the structure and thermotropic phase behavior of hydrated bilayers composed of DPPC and of its dialkyl, 1-acyl 2-alkyl and 1-alkyl 2-acyl analogs.

MATERIALS AND METHODS

Unlabeled and chain-perdeuterated samples of DPPC were synthesized and purified using previously published methods (see Lewis and McElhaney, 1985, 1992b; Lewis et al., 1987). The chain-perdeuterated palmitic acid used in the synthesis was obtained from MSD Isotopes (Montréal, Québec). 1,2-Hexadecyl-*sn*-glycero-3-phosphorylcholine (DHPC), 1-palmitoyl, 2-hexadecyl-*sn*-glycero-3-phosphorylcholine (PHPC), and 1-hexadecyl, 2-palmitoyl-*sn*-glycero-3-phosphorylcholine (HPPC) were obtained from other commercial sources (DHPC from Fluka, Buchs, Switzerland; PHPC and HPPC from Biochemisches Labor, Bern, Switzerland) and purified using the chromatographic procedures described by Lewis and McElhaney (1985). Lipid samples were prepared for the DSC measurements as follows. Lipid (3–4 mg) was dispersed in 0.75 ml of water at temperatures near 60°C, and 0.5-ml aliquots of this dispersion were analyzed in a Hart high-sensitivity differential scanning calorimeter (Hart Scientific, Provo, Utah) operating in the heating and cooling modes at scan rates near 15°C/h. Lipid concentrations were determined by the phosphate assay procedure described by Raheja et al. (1973). Lipid samples were prepared for FTIR spectroscopy as follows. Dry lipid (3–4 mg) was hydrated by the addition of 50 μl of D_2O (or H_2O as appropriate) followed by vigorous vortexing at temperatures near 60°C. This dispersion was then squeezed between the CaF_2 windows of a heatable liquid cell (equipped with a teflon spacer) to form 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 an external, computer-controlled circulating water bath. 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 and plotted with software supplied by Digilab and other computer programs obtained from the National Research Council of Canada and Microcal Software (Northampton, MA). In the analysis of the contours of the C=O stretching and CH_2 scissoring bands of these lipids, Fourier self-deconvolution was used to obtain accurate estimates of the peak frequencies of the component bands. Typically, the C=O stretching bands observed in the gel and liquid-crystalline states were deconvolved using bandwidth parameters (18 and 20 cm^{-1}, respectively) and band-narrowing factors (1.8–2) as defined by the software package supplied with the Digilab FTS-40 instrument. In the case of the CH_2 scissoring bands, the Fourier deconvolution procedures used to obtain peak maxima estimates were performed using similar band-narrowing factors, but with bandwidth parameters ranging from 7 to 10. Under the conditions of these experiments, band-narrowing factors of up to 2.2 could be employed without causing significant distortions of the deconvolved spectra. 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 component bands with the aid of standard nonlinear least-squares minimization procedures. The peak frequencies returned by Fourier self-deconvolution were used as starting estimates, and each band was simulated by a Gaussian-Lorentzian function, for which best-fit estimates of band shape were achieved with approximately 70% Gaussian contribution.

RESULTS

DSC heating and cooling thermograms of aqueous dispersions of the four lipids studied are shown in Fig. 1. With DHPC, PHPC, and HPPC, the observed thermotropic behavior was unaffected by prolonged low-temperature incu-

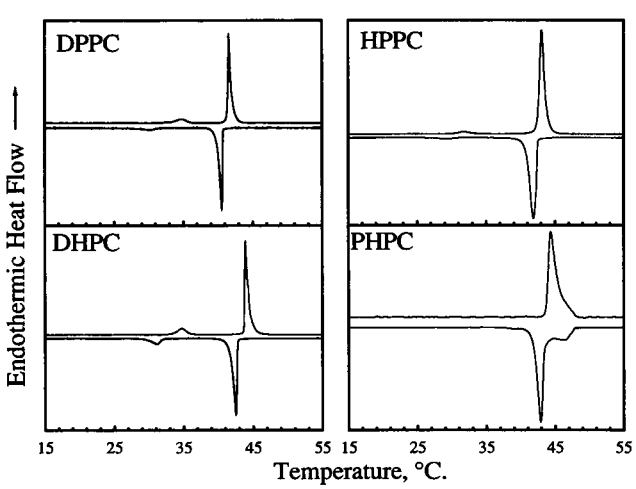

FIGURE 1 High sensitivity heating and cooling DSC thermograms exhibited by DPPC, HPPC, PHPC, and DHPC. The thermograms shown were acquired at a scan rate of 10°C/h^{-1} and, with the exception of that exhibited by DPPC, are essentially unaffected by prolonged low-temperature incubation.

bation. With DPPC, however, prolonged incubation at temperatures near 0–4°C results in the formation of the so-called subgel (lamellar crystalline, L_c) phase which, depending upon the length of the incubation period, reconverts to the normal lamellar gel (L_β) phase when heated to temperatures between 13°C and 24°C. This particular aspect of the behavior of aqueous DPPC bilayers has been intensively studied in this and other laboratories (see Fuldner, 1981; Lewis et al., 1987; Tristram-Nagle et al., 1987, 1994; Lewis and McElhaney, 1990, 1992b, and references cited therein) and, being peripheral to the focus of this work, will not be discussed further here. With DHPC we also observed a very weakly energetic transition (≤0.2 kcal/mol) near 5–6°C (see Fig. 2). This thermotropic event has been detected in previous DSC and x-ray diffraction studies of DHPC (Kim et al., 1987a,b; Lohner et al., 1987). However, those studies failed to identify the structural basis of that phenomenon, although they indicated that it does not involve a major structural rearrangement of the DHPC bilayer.

The DSC thermograms presented in Fig. 1 show that each of these lipids exhibits a highly cooperative, relatively energetic phase transition at temperatures between 41°C and 45°C (for details see Table 1). A combination of our FTIR spectroscopic studies (see below) and previous studies of these lipids using other techniques (Kim et al., 1987a,b; Laggner et al., 1987; Lohner et al., 1987; Haas et al., 1990) clearly indicate that these events are lamellar gel/liquid-crystalline phase transitions. An examination of the thermodynamic characteristics of these chain-melting phase transitions (see Table 1) indicates that replacement of an ester-linked hydrocarbon chain with the corresponding ether-linked counterpart results in small increases the temperature (DPPC < HPPC < DHPC < PHPC) and enthalpy

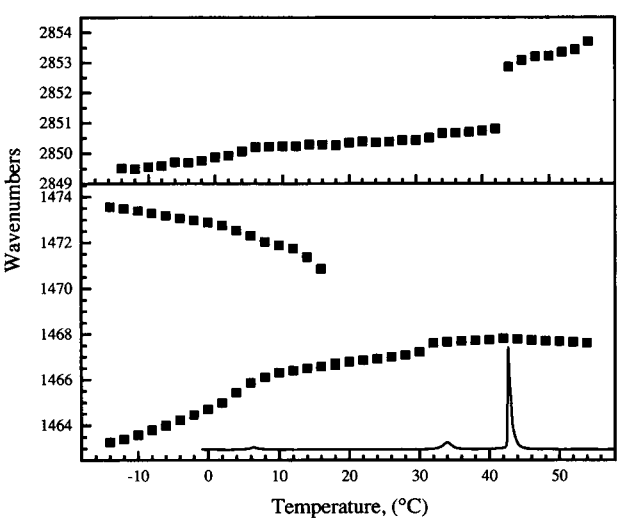

FIGURE 2 Temperature dependence of the CH_2 symmetric stretching (*top*) and CH_2 scissoring bands (*bottom*) bands of DHPC. To facilitate comparison of these data with the DSC results, a DSC heating thermogram of the lipid is also shown as an inset of the bottom panel.

TABLE 1 Thermodynamic characterization of the phase transitions exhibited by DPPC and its monoalkyl and dialkyl analogs

Sample	Transition type*	Transition temp‡ (°C)	Enthalpy change (kcal/mol)
Hydrocarbon chain melting phase transitions			
DPPC	P_β'/L_α	41.6	7.7
HPPC	L_β/L_α	43.2	7.9
PHPC	$L_{\beta i}/L_\alpha$	44.4	8.6
DHPC	L_β/L_α	43.9	8.0
Gel/gel phase transitions			
DPPC	L_β'/P_β'	34.5	≅1
HPPC	$L_{\beta i}/L_\beta$	32.0	≅1
PHPC	—	—	—
DHPC	$L_{\beta i}/L_\beta$	34.8	≅1

* Assigned on the basis of previously published x-ray diffraction studies (Kim et al., 1987a,b).
‡ Determined from the DSC heating thermograms.

of the gel/liquid-crystalline phase transition (DPPC < HPPC ≅ DHPC < PHPC). This observation was not unexpected, because similar results were observed in most studies in which ester-linked lipids are compared with their ether-linked counterparts (see Lewis and McElhaney, 1992a, and references cited therein). Interestingly, the data shown in Table 1 seem to suggest that the thermodynamic properties of these hydrocarbon chain-melting phase transitions may not necessarily be correlated with the number of ether bonds present, because the temperature and enthalpy of the phase transition exhibited by PHPC are both greater than that exhibited by DHPC. However, one should note that PHPC is the only one of these four lipids that does not exhibit a distinct and identifiable gel/gel phase transition before the melting of its hydrocarbon chains, and it is also the only one that exhibits a biphasic cooling exotherm at temperatures near the freezing point of its lipid hydrocarbon chains (see Fig. 1). Thus, it is possible that the higher T_m and ΔH values associated with the hydrocarbon chain-melting phase transition of this particular lipid may be the result of the simultaneous occurrence of the structural rearrangements that occur as distinct gel/gel and gel/liquid-crystalline phase transitions in the other lipids.

Fig. 1 also shows that with the exception of PHPC, the gel/liquid-crystalline phase transitions of these lipids are preceded by a gel/gel phase transition that is considerably less energetic and occurs some 7–10°C below the chain-melting phase transition. For DPPC, this gel/gel phase transition is the well-characterized pretransition at which changes in hydrocarbon chain tilt and headgroup mobility are known to occur (for more detailed discussions of the structural rearrangements occurring at the pretransition of DPPC and other *n*-saturated diacyl phosphatidylcholines (PCs), see Lewis and McElhaney, 1992a, and references cited therein). With DHPC and HPPC, however, x-ray diffraction studies have shown that their gel/gel phase transitions actually involve the conversion of a hydrocarbon chain-interdigitated lamellar gel phase (i.e., a $L_{\beta i}$ phase) to

a noninterdigitated lamellar gel (L_β) phase (Kim et al., 1987a,b; Laggner et al., 1987; Lohner et al., 1987). With PHPC, however, this event appears to be concomitant with the hydrocarbon chain-melting phase transition that occurs at temperatures near 44°C. Despite the fundamental differences in the nature of the pretransition of DPPC and the gel/gel phase transitions of the other lipids, however, the energetics of these structurally different processes seem to be very similar (see Table 1).

The top panel in Fig. 2 shows the temperature-dependent changes in the frequency of the methylene symmetric stretching ($_s\upsilon_{ch2}$) band exhibited by DHPC dispersions. A similar pattern of temperature-dependent changes in $_s\upsilon_{ch2}$ band frequency is exhibited by each of the lipids studied here. For each of these lipids, the sharp, highly energetic phase transitions detected by DSC are accompanied by discontinuous increases ($\cong$2 cm^{-1}) in their $_s\upsilon_{ch2}$ band maxima. This particular spectroscopic feature is characteristic of the increase in hydrocarbon chain conformational disorder that occurs when all-*trans* polymethylene chains melt (Snyder, 1967) and therefore clearly identifies the main thermotropic phase transitions of these lipids as hydrocarbon chain-melting phase transitions (for a recent review of the IR spectroscopic characteristics of lipid hydrocarbon chain-melting phase transitions, see Lewis and McElhaney, 1996). We also note that during the weakly endothermic transitions preceding the hydrocarbon chain-melting phase transitions of these lipids, their $_s\upsilon_{ch2}$ bands remain fairly sharp (not shown here), and their frequencies remain relatively low ($\leq$2850 cm^{-1}) and essentially unchanged. These observations indicate that, as expected, these weakly energetic transitions are solid-phase events (i.e., gel/gel transitions) between lipid structures with all-*trans* hydrocarbon chains, resulting in little or no change in hydrocarbon chain conformational order.

The bottom panel in Fig. 2 shows the temperature-dependent changes in the frequencies of the CH_2 scissoring vibration bands of DHPC, changes that are typical of those exhibited by the four lipids studied. At temperatures above T_m, DHPC, PHPC, HPPC, and DPPC all exhibit relatively broad CH_2 scissoring vibration bands near 1468 cm^{-1}, which sharpen significantly when cooled to gel-phase temperatures. Moreover, at temperatures just below T_m (i.e., up to 20°C below T_m), the CH_2 scissoring band appears as a single band near 1467 cm^{-1}, and upon further cooling, it splits into two components that are initially centered near 1466 and 1471 cm^{-1}. With all of these lipids the first appearance of this splitting (i.e., factor group, correlation field, or crystal field splitting) is not associated with any observable phase transition, and the peak frequencies of the component bands continue to drift apart with further decreases in temperature. This correlation-field splitting of the CH_2 scissoring bands indicates that in the gel phases of these lipids, the all-*trans* polymethylene chains of these lipids spontaneously assemble into subcells with perpendicularly packed zigzag planes (most probably orthorhombic perpendicular ($O\perp$) subcells) once reorientational fluctuations are sufficiently damped by cooling to low temperatures (Snyder, 1961, 1979). Furthermore, the observation that the magnitude of the splitting (i.e., the absolute difference between the frequency maxima of the two components) increases upon further cooling indicates that the mean size of the domains of orthorhombically packed hydrocarbon chains increases as the temperature decreases (Snyder et al., 1992). It is also noteworthy that the increase in the observed correlation field splitting is not a continuous function of temperature, and that a sharp change in the magnitude of this parameter occurs at temperatures near 5°C (see Fig. 2). A similar pattern of spectroscopic change was observed in recent studies involving long-chain PC bilayers and mixtures thereof (Mendelsohn et al., 1995; Snyder et al., submitted for publication). The latter authors attribute this behavior to thermally induced interconversions between two different gel phases, both with orthorhombically packed hydrocarbon chains (designated G_o and G_d; see Mendelsohn et al., 1995; Snyder et al., submitted for publication). Interestingly, with DHPC this thermally induced change in the magnitude of the correlation field splitting coincides with the calorimetrically detectable but weakly endothermic event that was alluded to earlier. The latter is centered near 5°C (see Fig. 2) and has been observed by us (this work) and by others (see Kim et al., 1987a,b; Lohner et al., 1987). With DPPC bilayers, correlation field splitting of the CH_2 scissoring bands has previously been observed in low-temperature and in high-pressure studies of the gel state (Cameron and Mantsch, 1982; Wong and Mantsch, 1985; Siminovitch et al., 1987a; Lewis and McElhaney, 1990, 1992b). In high-pressure FTIR spectroscopic studies of DHPC, the observed correlation field splitting was very strong (Siminovitch et al., 1987a), and it has been suggested that this may actually be a signature of chain-interdigitated lipid bilayers (Siminovitch et al., 1987b).

Despite the overall similarities of the spectroscopic data described above, there are subtle but structurally significant differences between many facets of their infrared spectra. For example, although the CH_2 scissoring bands of each of these lipids split into two components when these lipids are cooled to temperatures well below T_m, the distribution of absorbance intensity between these two components is not the same for each lipid (see Fig. 3 and summary in Table 2). Most probably this observation is the result of nonideal packing of the lipid hydrocarbon chains in the $O\perp$ subcells that they form (see Smith, 1953; Snyder, 1961). It has been determined that for an $O\perp$ subcell, the ratio of the integrated intensities of these components is related to the angle (θ) between the zigzag plane of the hydrocarbon chain and the *a*-axis of the crystallographic unit cell (for details, see Snyder, 1961). Consequently, it is possible to use these intensity ratios to semiquantitatively interpret our results in terms of deviations from ideal $O\perp$ packing. With ideally packed $O\perp$ subcells the integrated intensities of the high- and low-frequency components are expected to be equal, and this translates into θ being equal to 45° (see Smith,

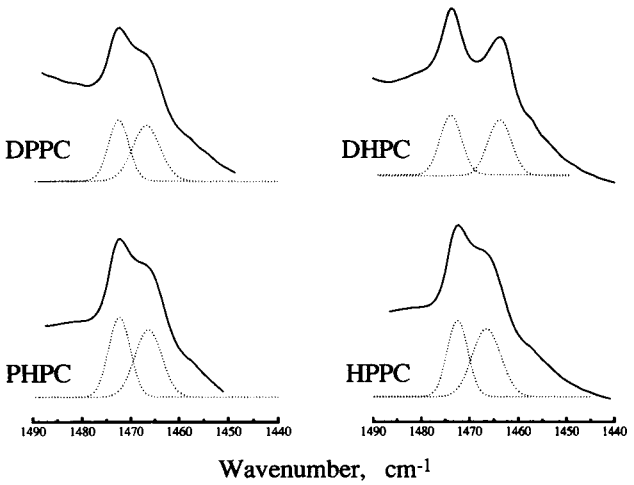

FIGURE 3 Comparison of the contours of the CH_2 scissoring bands of DPPC, PHPC, HPPC, and DHPC observed near −20°C. The observed absorbance spectra are represented by the solid lines, and the dotted lines represent our estimates of the contours of the subcomponents.

1953; Snyder, 1961). Our analyses (see summary in Table 2) suggest that the hydrocarbon chains of DHPC adopt a near-ideal form of O⊥ packing, whereas the packing formats adopted by PHPC, HPPC, and DPPC, respectively, are progressively distorted from that ideal. It is generally observed that ideally packed O⊥ subcells are more commonly found in compounds with fairly long aliphatic chains and relatively small terminal groups, and deviations from ideal O⊥ packing are normally attributed to the distortions induced by the packing requirements of larger end groups (Smith, 1953). With DHPC bilayers, however, end-group effects due to the ether oxygens seem to be minimal, and this probably enables these lipid molecules to spontaneously adopt conformations that are conducive to near-ideal O⊥ packing of the hydrocarbon chains. With DPPC, HPPC, and PHPC, however, the greater steric bulk and polarity of the ester carbonyl oxygens may impose packing requirements that cannot be accommodated without distorting the O⊥ packing of hydrocarbon chains. Moreover, given our evidence for significant differences between the conformations of DPPC and DHPC, PHPC, or HPPC (see below), the data presented above also suggest that the accommodation of the steric bulk and polarity of two ester carbonyl oxygens of DPPC may be severe enough to induce conformational changes in the phospholipid molecule as a whole.

TABLE 2 Comparison of the I_{1466}/I_{1472} ratios of the CH_2 scissoring bands of DPPC, HPPC, PHPC, and DHPC

Sample	I_{1466}/I_{1472}*	$\theta^{\ddagger}$	$\Delta\theta^{\S}$
DPPC	1.25	38.7°	6.3°
HPPC	1.22	39.2°	5.8°
PHPC	1.08	42.8°	2.2°
DHPC	1.005	44.9°	0.1°

* Ratio of the integrated intensity of the low- and high-frequency components of the CH_2 scissoring band. The ratios were determined from area estimates obtained by a combination of Fourier deconvolution and curve fitting.

‡ Angles estimated according to the method of Snyder (1961) and rounded to one decimal place. Because of the errors in area estimation, the accuracy of these estimates is expected to be ±0.7°.

§ Difference between the observed θ and that expected of an ideally packed orthorhombic perpendicular subcell.

An examination of the α-methylene scissoring region of the infrared spectra of these lipids also reveals other subtle but structurally significant spectroscopic features. As illustrated in Fig. 4 (*right*), DPPC, HPPC, and PHPC each exhibit moderately absorptive α-methylene scissoring bands near 1418 cm^{-1}. Interestingly, however, recent studies using specifically chain-perdeuterated *n*-saturated 1-acyl, 2-acyl PCs have demonstrated that the observable α-methylene scissoring absorptions bands of those lipids arise exclusively from the scissoring vibrations of α-methylene groups on the *sn*1 fatty acyl chain (Lewis and McElhaney, 1992b, 1993). This point is vividly illustrated in Fig. 4 (*left*), which compares the α-methylene bands observed in the gel phases of unlabeled, *sn*1 chain-perdeuterated, and *sn*2-chain-perdeuterated samples of DPPC. With unlabeled and *sn*2 chain-perdeuterated DPPC (spectra C and B, respectively), α-methylene scissoring absorptions near 1418 cm^{-1} are easily discernible, whereas with *sn*1 chain-perdeuterated DPPC (spectrum A), infrared absorption near 1418 cm^{-1} is almost completely suppressed. It has been suggested that such observations may be a reflection of conformational differences between the *sn*1 and *sn*2 α-methylene groups of the lipids (see Lewis and McElhaney, 1992b, 1993), a suggestion that is compatible with the known (or generally assumed) conformational inequivalence between the *sn*1 and *sn*2 fatty acyl chains of such lipids (see Pearson and Pascher, 1979; Hitchcock et al., 1974; Hauser et al., 1981, 1988). Given this, the fact that the *sn*2 and *sn*1 α-methylene groups of HPPC and PHPC, respectively, each exhibit scis-

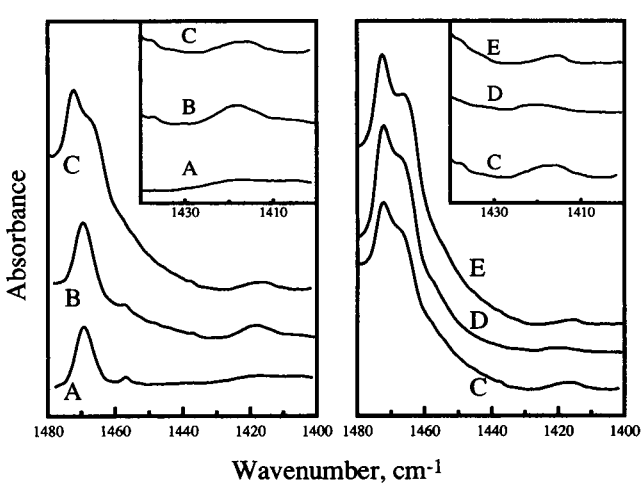

FIGURE 4 The CH_2 deformation regions of the infrared spectra of DPPC and its monoalkylated analogs. The absorbance spectra shown were acquired at −20°C. (*A*) DPPC (*sn*1-d31). (*B*) DPPC (*sn*2-d31). (*C*) DPPC (unlabeled). (*D*) PHPC. (*E*) HPPC.

soring absorptions comparable to that exhibited by the *sn*1 α-methylene group of DPPC, suggests that the conformations of the palmitoyl chains located at the *sn*1 position of DPPC, the *sn*1 position of PHPC, and the *sn*2 position of HPPC are similar. With long-chain glycerolipids, it is virtually impossible to construct a bilayer in which acyl chains located at the *sn*1 and *sn*2 positions of the glycerol backbone have similar conformations while the glycerol backbone retains a conformation comparable to that observed in PC and PE crystals (see Hitchcock et al., 1974; Pearson and Pascher, 1979) or inferred for hydrated PC and PE bilayers (i.e., perpendicular to the bilayer plane; see Hauser et al., 1981, 1988). Thus these results imply that the conformations of the glycerol backbones of the acyl-alkyl analogs of DPPC may actually differ from that of DPPC. The implications of this conclusion will be further explored in the Discussion.

One of the more striking features of these FTIR spectroscopic studies emerges from an analysis of the contours of the carbonyl stretching ($\upsilon_{C=O}$) bands of these lipids (see Fig. 5 and Table 3). In both the lamellar liquid-crystalline (L_α) and L_β phases, DPPC exhibits a broad $\upsilon_{C=O}$ absorption band centered near 1733 cm^{-1}, which appears to be a summation of components centered near 1742 and 1727 cm^{-1}. It has been suggested that the high- and low-frequency components may be attributable to differential infrared absorption by subpopulations of free and hydrogen-bonded ester carbonyl groups, respectively (see Blume et al., 1988). Recent studies have also shown that each of these components is in turn a summation of contributions from both the *sn*1 and *sn*2 ester carbonyl groups (Blume et al., 1988; Lewis and McElhaney, 1992b, 1993; Lewis et al., 1994b). As is typical of most 1,2-diacyl glycerolipids studied so far, the relative sizes of these populations are dependent on both the phase state of the lipid and the absolute temperature. The data presented in Fig. 5 and Table 3 also indicate that the properties of the $\upsilon_{C=O}$ absorption bands of both PHPC and HPPC differ from those of DPPC in two significant aspects. First, the $\upsilon_{C=O}$ bands of both HPPC and PHPC exhibit their absorption maxima near 1726 cm^{-1}, some 7 cm^{-1} lower than that of DPPC. From an examination of the substructure of the C=O bands of these lipids (see below), it is obvious that the above is primarily the result of the appearance of an additional low-frequency C=O band near 1710 cm^{-1}, presumably at the expense of one or both of the other two bands that are typical of the 1,2-diacyl PCs (see Fig. 5). Thus, given that ester carbonyl groups located in more polar and/or more hydrated environments tend to absorb infrared radiation at lower frequencies (see Mushayakarara et al., 1986; Blume et al., 1988, and references cited therein), the appearance of this additional low-frequency band in the IR spectra of the two acyl-alkyl analogs suggests that substitution of any one of the acyl chains of DPPC with the corresponding alkyl group results in an increase in the polarity and/or hydration of the environment around the remaining ester carbonyl group. This surprising conclusion seems counterintuitive when viewed against the fact that ester linkages are nominally more polar than ether linkages. Second, the $\upsilon_{C=O}$ absorption band of both PHPC and HPPC seems to be resolvable into three components that are centered near 1740, 1725, and 1710 cm^{-1} (see Fig. 5 and Table 3). Unlike DPPC and other 1,2-diacyl glycerolipids, however, PHPC and HPPC each contain a single ester carbonyl group, and as a result these subcomponents must be attributable to differential infrared absorptions by subpopulations in which the local environments of the single ester carbonyl group differ. It is therefore possible that these components arise from free ester carbonyl groups (≅1740 cm^{-1}) and two separate populations of hydrogen-bonded carbonyl groups (≅1725 and 1710 cm^{-1}). Thus hydrated acyl-alkyl PC bilayers may each contain a population of hydrogen-bonded ester carbonyl groups (≅1710 cm^{-1}) that is not normally present in typical 1,2-diacyl PCs. This observation and others described here have important structural implications, which will be examined later.

TABLE 3 Infrared absorption maxima* of the $\upsilon_{C=O}$ bands of DPPC and its acyl-alkyl analogs

Sample	Observed (cm^{-1})	Subcomponents (cm^{-1})‡
DPPC	1733	1742, 1727
PHPC	1726	1740, 1725, 1710
HPPC	1726	1739, 1725, 1712

* Data obtained from spectra recorded in the L_α phase of each lipid and rounded to the nearest wavenumber.

‡ Estimated by a combination of Fourier deconvolution and curve fitting.

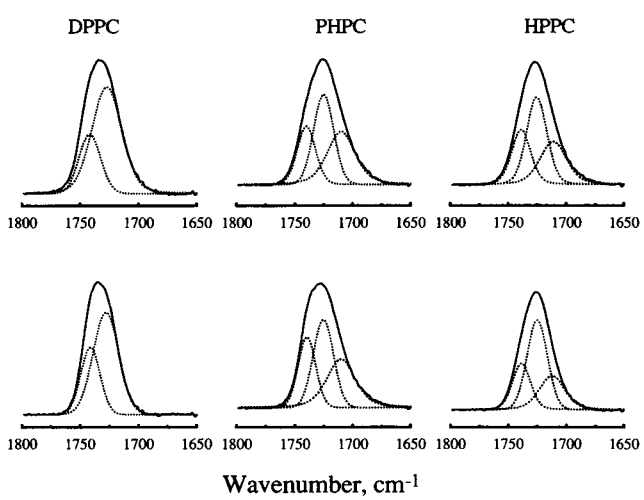

FIGURE 5 The C=O stretching bands of DPPC and its monoalkylated analogs. Absorbance spectra are presented for the gel (*bottom*) and liquid-crystalline (*top*) phases. The solid lines represent the spectra actually acquired, and the broken lines represent our estimates of the underlying components.

The right panel in Fig. 6 shows the antisymmetric phosphate stretching ($_{as}\upsilon_{PO2}^-$) regions of the infrared spectra of H_2O dispersions of DPPC, HPPC, PHPC, and DHPC at temperatures below their respective T_m's. At these temper-

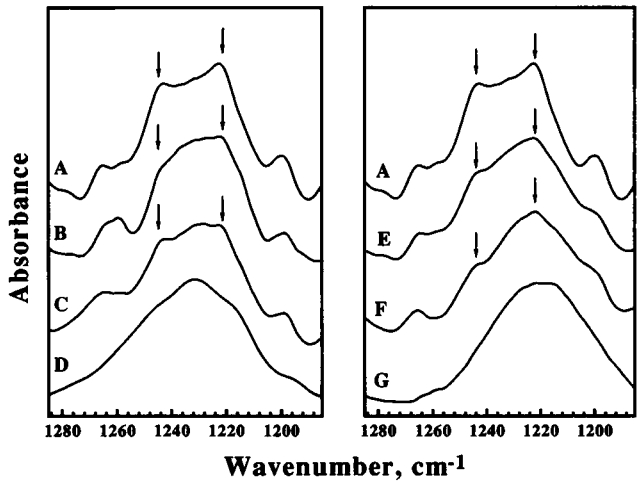

FIGURE 6 The $_{as}\upsilon_{PO2}^-$ regions of the infrared spectra of unlabeled, specifically chain perdeuterated, and chain perdeuterated DPPC and of the 1-acyl, 2-alkyl, 1-alkyl, 2-acyl, and 1,2-dialkyl analogs. The absorbance spectra shown were acquired in the gel phase of these lipids and have been normalized relative to the integrated intensities of the underlying $_{as}\upsilon_{PO2}^-$ band. The arrows indicate the absorptions attributable to the CH_2 wagging band progressions. (*A*) DPPC (unlabeled). (*B*) DPPC (*sn1*-d31). (*C*) DPPC (*sn2*-d31). (*D*) DPPC (d62). (*E*) PHPC. (*F*) HPPC. (*G*) DHPC.

atures the relatively weak absorption bands arising from the CH_2 wagging band progressions of the lipid all-*trans* hydrocarbon chains are clearly discernible over the broader contours of the $_{as}\upsilon_{PO2}^-$ bands of these lipids. A close examination of these band progressions reveals a number of interesting and structurally relevant points. First, in marked contrast to DPPC, HPPC, and PHPC, the band progression absorptions exhibited by DHPC are barely discernible. This observation suggests that the infrared absorption CH_2 wagging band progressions of the ether-linked hydrocarbon chains are probably weak enough to be ignored in these considerations (i.e., we can assume that absorption bands exhibited by the 1-alkyl, 2-acyl and 1-acyl, 2-alkyl PCs arise exclusively from the band progressions of the acyl-linked hydrocarbon chain). Second, the intensities of the band progression absorptions by the 1-alkyl 2-acyl and the 1-acyl 2-alkyl PCs seem to be comparable, despite the inequivalent locations of the palmitoyl chains on the glycerol backbones of the two lipids. This observation is particularly interesting when viewed against the results of the parallel study of the band progressions exhibited by DPPC and its chain-deuterated analogs (see Fig. 6, *left*). As expected, perdeuteration of the two hydrocarbon chains of DPPC effectively suppresses CH_2 wagging band progression absorptions (spectrum D), whereas perdeuteration of a single chain significantly attenuates the intensities of the band progressions exhibited by both the *sn*1 and *sn*2 chain-perdeuterated analogs (spectra B and C, respectively). However, these data also indicate that the *sn*2 chain-perdeuterated lipid (spectrum C) exhibits stronger CH_2 wagging band progression absorptions than does the corresponding *sn*1 chain-perdeuterated lipid (spectrum B). With all-*trans* fatty acyl chains, the intensity of the CH_2 wagging band progressions is enhanced by both an increase in acyl chain length and by the coupling of the methylene group vibrations to those of the C=O group (Jones and Sandorfy, 1956). Thus, if one assumes that the gel-phase conformations of 1,2-diacyl glycerolipids such as DPPC are comparable to those observed in single-crystal x-ray studies of PC and PE (see Hitchcock et al., 1974; Pearson and Pascher, 1979) or inferred from x-ray and NMR studies of hydrated lipids (Hauser et al., 1981, 1988), then the conformation of the *sn*1 chain should be very favorable to enhanced infrared absorption by the CH_2 wagging band progressions of that chain. This is because the expected conformation of the *sn*1 chain favors the maximization of the all-*trans* length of that chain as well as the vibrational coupling between the all-*trans* methylenes and the ester C=O group. With the *sn*2 fatty acyl chain, however, one expects that there will be a β-γ bend in the hydrocarbon chain. This should significantly attenuate the CH_2 wagging band progression of that fatty acyl chain because it shortens the maximum possible all-*trans* polymethylene length and uncouples (or interferes with) interactions between the all-*trans* methylenes and the ester C=O group. Given these arguments, the greater intensity of CH_2 band progressions exhibited by *sn*2 chain-perdeuterated DPPC can be easily rationalized by the conformational inequivalence of the *sn*1 and *sn*2 fatty acyl chain of that lipid. However, the fact that similar differences between the absorption intensities of the CH_2 wagging band progressions of the *sn*1 acyl chain of PHPC and *sn*2 acyl chain of HPPC are not observed suggests the conformational inequivalence of the *sn*1 and *sn*2 acyl chains of DPPC is not mirrored by the *sn*1 and *sn*2 palmitoyl chains of PHPC and HPPC, respectively. This observation lends further support to the suggestion that the conformation of the glycerol backbones of 1-acyl, 2-alkyl and 1-alkyl, 2-acyl PC analogs may differ from that of DPPC (see above).

The data presented in Table 4 are the $_{as}\upsilon_{PO2}^-$ band maxima observed in the L_α phases of the four lipids studied. It is clear that these maxima decrease in the order DPPC > PHPC > HPPC > DHPC. Given that a decrease in the frequency of the $_{as}\upsilon_{PO2}^-$ band is generally indicative of the location of the lipid phosphate polar headgroups in more polar environments (see Wong and Mantsch, 1988, and references cited therein), this observation suggests that the substitution of one or both of the ester-linked hydrocarbon chains of DPPC with the corresponding ether-linked analog

TABLE 4 Infrared absorption maxima* of the $_{as}\upsilon_{PO2}^-$ bands of DPPC, HPPC, PHPC and DHPC

Sample	Frequency (cm^{-1})
DPPC	1231
PHPC	1229
HPPC	1226
DHPC	1220

* Data obtained from spectra recorded in the L_α phase of each lipid and rounded to the nearest wavenumber.

results in an increase in the polarity of the environment around the phosphate headgroup of the lipid. This conclusion also seems counterintuitive when viewed against the fact that ester linkages are nominally more polar than ether linkages. Thus these frequency shifts are almost certainly a reflection of changes in headgroup hydration (or hydrogen bonding) induced by changes in the polar/apolar interfaces of lipid bilayers formed by these alkyl group-containing PCs. Our attempts to examine the phase-state dependences of the $_{as}\upsilon_{PO2}^{-}$ band maxima of these lipids were impeded by interference from the CH_2 wagging band progressions that appear in the gel state. However, because such interference was minimal with DHPC (see above), it was possible to acquire such data with that lipid and to compare it with a comparable study of chain-perdeuterated DPPC, where the band progressions have been eliminated by perdeuteration. The results of that study indicate that the peak frequencies of the $_{as}\upsilon_{PO2}^{-}$ band of DPPC are essentially insensitive to changes in temperature and lipid phase state, whereas with DHPC, the $_{as}\upsilon_{PO2}^{-}$ band exhibits a discontinuous increase in frequency ($\cong$2–3 cm^{-1}) at the gel/gel phase transition observed near 35°C (see Fig. 7). This result clearly indicates that the gel/gel transition of DHPC is a structurally distinct phenomenon from the pretransition of DPPC. Our results suggest that in the heating mode, this transition results in a small decrease in the polarity of the phosphate headgroup and may therefore be indicative of a change in the hydration/or hydrogen bonding interaction of that moiety. Previous work has shown that at the gel/gel transition of DHPC a conversion from a chain-interdigitated lamellar gel phase to a more typical noninterdigitated lamellar gel phase (i.e., a $L_{\beta i}/L_{\beta}$ phase transition) occurs (Kim et al., 1987a,b; Laggner et al., 1987; Lohner et al., 1987). Given this, one can probably explain the spectroscopically detectable decrease in the polarity of the phosphate environment of DHPC by a decrease in the accessibility of water to the headgroup phosphate of DHPC at the $L_{\beta i}/L_{\beta}$ phase transition. In the $L_{\beta i}$ phase the lowered surface density of lipid headgroups (one headgroup per four hydrocarbon chains) will reduce steric crowding of the headgroup phosphates, thereby making the headgroup phosphate more accessible to water. At temperatures above the $L_{\beta i}/L_{\beta}$ phase transition, the increase in the surface density of headgroup phosphates (to one headgroup per two hydrocarbon chains) will increase steric crowding of the headgroup phosphates and, in turn, decrease their accessibility to water.

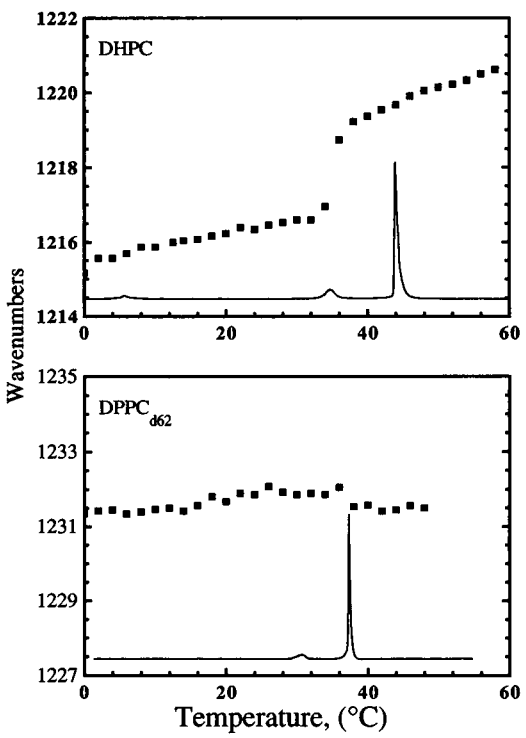

FIGURE 7 Temperature dependence of the $_{as}\upsilon_{PO2}^{-}$ bands maxima of DHPC (*top*) and chain perdeuterated DPPC (*bottom*). To facilitate comparison of these data with the DSC results, DSC heating thermograms of the respective lipids are shown as insets of each panel.

DISCUSSION

This study vividly demonstrates that even small changes in the chemical structure of the polar/apolar interfacial region of a lipid molecule can markedly affect many aspects of the lipid-lipid and lipid-water interactions that occur in bilayer assemblies. Together, our results and previously published work (see Kim et al., 1987a,b; Lohner et al., 1987; Laggner et al., 1987; Haas et al., 1990, and references cited therein) indicate that substitution of an acyl chain with an appropriate alkyl chain induces hydrocarbon chain interdigitation in the gel phase and may also increase the polarity of the local environments of both the headgroup phosphate moiety and of any remaining ester carbonyl groups at the bilayer polar/apolar interface. That substitution of an alkyl chain for an acyl chain should exert such effects seems surprising when one considers that such changes are not expected to drastically alter the "chemical character" of the interfacial regions of these PC analogs and that ether linkages are considered to be less polar than ester linkages. Currently, the reason why the substitution of an acyl chain with an alkyl chain should exert these particular effects is not well understood. However, our systematic spectroscopic investigations of these PC analogs may provide a means whereby the basis of these effects can be explained. Specifically, a comparison of the α-methylene scissoring bands and the CH_2 wagging band progressions of DPPC, PHPC, and HPPC with those of the specifically chain-perdeuterated analogs of DPPC suggest that the *sn*1-palmitoyl chain of PHPC and the *sn*2-palmitoyl chain of HPPC may actually adopt similar conformations and that their conformations may well be comparable to that expected of the *sn*1-palmitoyl chain of DPPC. Using molecular models, we find it virtually impossible to form stable HPPC and PHPC bilayers that satisfy such conformational requirements while simultaneously retaining the glycerol backbone in a conformation similar to that determined in

single-crystal x-ray studies of PC and PE (see Hitchcock et al., 1974; Pearson and Pascher, 1979) or inferred from x-ray diffraction and NMR studies of hydrated PC and PE bilayers (see Hauser et al., 1981, 1988). Given this, we suggest the mechanism through which substitution of an acyl chain with its alkyl counterpart alters the bilayer physical properties of these DPPC analogs is the induction of a conformational change in the glycerol backbone of the lipid.

Our studies using molecular models also suggest that the formation of stable bilayers that meet the conformational requirements suggested by our FTIR spectroscopic data can be relatively simple if the lipid glycerol backbone were aligned parallel to the bilayer surface instead of along the bilayer normal. The suggestion that the glycerol backbones of DHPC, PHPC, and HPPC actually adopt such a conformation is particularly attractive because it offers a rationale for a number of our experimental observations. First, the alignment of the glycerol backbone in such a manner will almost certainly result in an increase in the mean intermolecular spacing at the bilayer surface. Given the above and the presence of excess water, one can easily envisage that water access to various hydration sites on the phosphate headgroup would be enhanced because of decreased steric crowding. Enhanced phosphate hydration or hydrogen bonding to water will account for the fact that the ${}_{as}\upsilon_{PO2}^{-}$ band of DHPC is centered at a lower frequency than that of DPPC (see Table 4). Second, the conformational change alluded to above will probably allow both the *sn*1 and *sn*2 hydrocarbon chains to adopt fully extended conformations. Because of this, there would be a net increase in the all-*trans* length of the hydrocarbon chains, a result that should be reflected by higher hydrocarbon chain-melting phase transition temperatures. Third, because the net increase in hydrocarbon chain all-*trans* length will also be accompanied by an increase in interheadgroup spacing, there may also be an enhanced propensity to form chain-interdigitated gel phases. The latter suggestion is consistent with the results of recent studies which demonstrate that the increase in lipid-lipid spacing caused by the partitioning of small molecules into the interfacial regions of phospholipid bilayers can induce hydrocarbon chain interdigitation (see Simon and McIntosh, 1984; Wilkinson et al., 1987, and references cited therein). Finally, the conformational change in the lipid glycerol backbone and the concomitant increase in intermolecular spacing could also explain why there is an increase in the polarity of the local environment of the ester carbonyl groups. This is because an increase in intermolecular spacing should also reduce steric crowding in the interfacial region, thereby making the hydration sites on the ester carbonyl groups more accessible to water.

In principle, the suggestion that changes in the chemical structure and/or configuration of lipid polar/apolar interfaces may actually induce major conformational changes in lipid molecules should not be surprising. This is because the steric bulk and the electronic configuration of moieties located at or near bilayer polar/apolar interfaces each have specific geometric requirements for optimal packing interactions that may not necessarily be compatible with idealized packing interactions between the polar headgroups and between the hydrocarbon chains. Within any lipid bilayer the interactions that actually occur usually represent a compromise between the specific requirements of these three domains, and as a result chemical and or configurational changes in any one domain can easily have an impact upon the nature and strengths of the interactions between moieties in other domains. However, because of the pivotal location of the bilayer polar/apolar interface, changes in its chemical structure and/or configuration have the potential to affect lipid conformation and bilayer properties to an extent that may seem to be disproportionately larger than the magnitude of the chemical or configurational change itself. One should note, however, that the capacity to effect such changes will almost certainly be dependent upon the nature and the strengths of the interactions possible between the lipid polar headgroups and between its hydrocarbon chains. Thus, although our data suggest that the structural adjustments needed to accommodate the ester carbonyl oxygens of DPPC were sufficient to induce significant changes in lipid conformation, headgroup and interfacial hydration, and the preferred mode of hydrocarbon chain packing, it does not necessarily mean that such changes will always occur when the acyl chains of glycerolipid bilayers are substituted with their alkyl counterparts. This point is underscored by the fact that replacement of the acyl chains of all ethanolamine and the monoglycosyl glycerolipids studied so far with appropriate alkyl-linked counterparts seems not to induce hydrocarbon chain interdigitation in those lipid bilayers (see Seddon et al., 1984; Kuttenreich et al., 1988; Sen et al., 1990; Hing et al., 1991; Hinz et al., 1991).

Finally, these results also raise fundamental questions about whether dialkyl and/or alkyl-acyl glycerolipids are actually good models for their diacyl counterparts. In the case of these DPPC analogs, it is clear that the replacement of acyl chains with their alkyl counterparts alters both the preferred conformation of the lipid molecule and its preferred form of gel-state packing. Thus, in many respects, the structure and organization of hydrated DPPC bilayers may be sufficiently different from those of the mono- and dialkyl derivatives to make the latter poor models of DPPC. This conclusion seems consistent with the observation that HPPC is considerably more resistant to phospholipase A_2-catalyzed hydrolysis than is DPPC (unpublished observations from this laboratory). However, as noted above, it is not clear how generally applicable the inferences drawn from our experimental observations actually are. Indeed, considering that the propensity for forming chain-interdigitated structures will probably be attenuated when the hydrocarbon chains are shortened and headgroup-headgroup interactions are strengthened, one cannot be certain whether shorter chain PCs or longer chain PEs and MGDGs will exhibit comparable behavior. Nevertheless, the fact that even small changes in the chemical structure of lipid polar/apolar interfaces can have such wide-ranging effects on phospholipid conformation and packing underscores the

critical importance of this region of lipid assemblies. In view of the current shortage of data about this critical aspect of lipid behavior, it is evident that a full understanding of the principles underlying the phenomena observed will require more in-depth studies of how the effects of lipid interfacial structure are moderated by changes in hydrocarbon chain length and structure and by changes in the structure of the lipid polar headgroups. Such work is part of the ongoing investigations being pursued in this laboratory.

This work was supported by operating and major equipment grants from the Medical Research Council of Canada (RNM), major equipment grants from the Alberta Heritage Foundation for Medical Research (RNM), and by a grant from the Deutsche Forschungsgemeinschaft (WP, project Po 423/3-1).

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