

Calorimetric and Spectroscopic Studies of the Thermotropic Phase Behavior of the *n*-Saturated 1,2-Diacylphosphatidylglycerols

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ABSTRACT The polymorphic phase behavior of a homologous series of *n*-saturated 1,2-diacyl phosphatidylglycerols (PGs) was studied by differential scanning calorimetry and Fourier transform infrared and ^{31}P -nuclear magnetic resonance spectroscopy. When dispersed in aqueous media under physiologically relevant conditions, these compounds exhibit two thermotropic phase transitions that are structurally equivalent to the well-characterized pretransitions and gel/liquid-crystalline phase transitions exhibited by bilayers of the corresponding 1,2-diacyl phosphatidylcholines. Furthermore, when incubated at low temperatures, their gel phases spontaneously transform into one or more solid-like phases that appear to be highly ordered, quasicrystalline bilayers that are probably partially dehydrated. The quasicrystalline structures, which form upon short-term, low-temperature annealing of these lipids, are meta-stable with respect to more stable structures, to which they eventually transform upon prolonged low-temperature incubation. The rates of formation of the quasicrystalline phases of the PGs generally tend to decrease as hydrocarbon chain length increases, and PGs whose hydrocarbon chains contain an odd number of carbon atoms tend to be slower than those of neighboring even-numbered homologs. The calorimetric data also indicate that the quasicrystalline phases of these compounds become progressively less stable relative to both their gel and liquid-crystalline phases as the length of the hydrocarbon chain increases and that they decompose either to the liquid-crystalline phase (short- and medium-chain compounds) or to the normal gel phase (long-chain compounds) upon heating. The spectroscopic data indicate that although there is odd-even alternation in the structures of the quasicrystalline phases formed upon short-term low-temperature incubation of these compounds, the structural features of the stable quasicrystalline phases eventually formed are all similar. Furthermore, the degree of hydration and the nature of hydrogen bonding interactions in the headgroup and interfacial regions of these PG bilayers differ significantly from that observed in all other phospholipid bilayers studied so far. We suggest that many of the properties of PG bilayers can be rationalized by postulating that the glycerol moiety of the polar headgroup is directly involved in shielding the negative charges at the surface of the bilayer by means of hydration-like hydrogen bonding interactions with the phosphate moiety.

INTRODUCTION

Although zwitterionic and uncharged lipids are the predominant components of the lipids of biological membranes, anionic lipids are invariably present in substantial quantities. In eucaryotic membranes the latter tend to be localized predominantly in the inner surface of the lipid bilayer (for recent reviews see Lipowsky, 1995; Williamson and Schlegel, 1994). Numerous studies have shown that cell growth and various membrane functions have an absolute requirement for at least minimal quantities of anionic lipids (see van der Goot et al., 1993; Xia and Dowhan, 1995a; and references cited therein). Because anionic lipids impart a negative charge to the surfaces of lipid bilayers, it has been assumed that one of the specific functions of anionic lipids is the regulation of membrane lipid surface charge density (see Christiansson et al., 1985; Clementz and Christiansson, 1986; Clementz et al., 1987; and references cited therein). Indeed, lipid bilayer surface charge density can have major effects on the passive permeability of biological membranes

to ions and charged metabolites and on the activity of a number of membrane-bound enzymes and transport proteins (see Huunan-Seppalla, 1971; Schafer and Rowohl-Quisthoudt, 1975, 1976; McLaughlin et al., 1970; Theuvenet et al., 1976a,b; Wojtczaj and Nalecz, 1979; and references cited therein). Others have shown that membrane lipid surface charge density affects the growth, transbilayer membrane potential, and adenylate energy charge of *Acholeplasma laidlawii* cells, and that lipid surface charge density appears to be biosynthetically regulated in this organism (Christiansson et al., 1985; Clementz and Christiansson, 1986; Clementz et al., 1987). Moreover, we have shown that the *A. laidlawii* ($\text{Na}^+ + \text{Mg}^{2+}$)-ATPase requires modest levels of anionic lipid for maximum activity, but that the presence of high levels of anionic lipids can result in an irreversible loss of function (George et al., 1989). However, because some membrane-associated enzymes and transporters also appear to have requirements for specific anionic lipids (Yamazaki et al., 1988; Pinheiro and Watts, 1994; de Jongh et al., 1995; Xia and Dowhan, 1995b), it seems clear that aside from their role in regulating bilayer surface charge density, some anionic lipids must have more specific functions in biological membranes. The latter functions are poorly understood at present, and much more work needs to be done before the general and specific roles of anionic lipids in biological membranes are fully understood.

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Phosphatidylglycerol (PG) and phosphatidylserine (PS) are the predominant anionic lipids found in nature. PS is usually the major anionic lipid found in eucaryotic cell membranes, whereas PG is the predominant (and often the only) anionic lipid found in procaryotic cell membranes. The PGs are the most intensively studied class of naturally occurring anionic lipids, and the *n*-saturated 1,2-diacyl PGs are frequently used in biophysical studies of negatively charged model membrane systems (for examples, see Watts et al., 1978; Seelig et al., 1987; Macdonald et al., 1991; Heimburg and Biltonen, 1994; and references cited therein). Such studies indicate that the thermotropic phase behavior of PG bilayers is more sensitive to factors such as pH, ionic strength, and the presence of divalent cations than are zwitterionic phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (see Watts et al., 1978), and that surface charge can markedly affect the structure of such bilayers in both the gel and liquid-crystalline phases (Watts et al., 1981, and references cited therein). Nevertheless, when examined under physiologically relevant conditions (i.e., near neutral pH, 100–200 mM salt, low [\leq μ M] concentrations of divalent cations), their thermotropic phase behavior exhibits many similarities to that of the corresponding PCs (Watts et al., 1978; Watts and Marsh, 1981; Findlay and Barton, 1978), including the formation of so-called lamellar subgel phases upon incubation at low temperatures (Wilkinson and McIntosh, 1986; Blaurock and McIntosh, 1986). However, even under physiologically relevant conditions, the *n*-saturated 1,2-diacyl PGs can exhibit a more complex pattern of thermotropic phase behavior than the corresponding PCs, including the formation of one or more high-melting crystalline or quasi-crystalline lamellar phases (Theret et al., 1983; Boggs and Moscarello, 1984; Epan et al., 1992; Kodama et al., 1993). Interestingly, the formation of high-melting gel or crystalline phases has also been observed in studies of the interaction of PG bilayers with some proteins and peptides that interact with the polar surfaces of lipid bilayers (for examples, see Surewicz and Epan, 1986; Heimburg and Biltonen, 1994). However, because of the complexity of the intrinsic phase behavior of PG itself, it is difficult to accurately interpret such observations in the absence of more complete information about the structures of the phases that are formed in the presence and absence of those peptides or other additives. Because there is relatively little information about the nature and the structural basis of the complex phase behavior of PG bilayers, we have begun a broadly based study of the polymorphic phase behavior of PG bilayers and a characterization of the structural features of the various phases that they spontaneously form under physiologically relevant conditions, so that additional insight into the molecular basis of the structural and functional roles of PGs in biological membranes may be obtained. The results of our differential scanning calorimetry (DSC), 31 P-NMR, and Fourier transform infrared (FTIR) spectroscopic studies of a homologous series of *n*-saturated 1,2-diacyl PGs are presented here.

MATERIALS AND METHODS

The PGs used for this study were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The lipid dispersions used for DSC and 31 P-NMR spectroscopic studies were prepared by the vigorous vortexing of dry lipid samples in a buffer containing 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7.4) at temperatures well above the T_m (gel/liquid-crystalline phase transition temperature) of the lipids. A combination of Hart 7701 (Calorimetry Sciences Corporation, Provo, UT) and Microcal MC2 (Microcal, Northampton, MA) scanning calorimeters operating at scan rates near 10–15°C/h were used to record high-sensitivity heating and cooling thermograms of these lipids. Procedures for quantifying the lipid samples were the same as previously used in this laboratory (Lewis and McElhaney, 1985a). The data acquired were analyzed and plotted with Microcal Origin software (Microcal Software). 31 P-NMR spectra were recorded with a Varian Unity 300 spectrometer (Varian, Palo Alto, CA) operating at 121.42 MHz for 31 P. Spectra were recorded using single-pulse data acquisition techniques as described previously (Lewis et al., 1988). The spectra were subsequently plotted with Microcal Origin software. For the FTIR spectroscopic experiments, 2–3 mg of the dried lipid was dispersed in D₂O buffered with 100 mM phosphate (pH 7.4) to observe the CH₂ stretching, CH₂ scissoring, and C=O stretching bands, or in H₂O buffered with 50 mM Tris, 100 mM NaCl, 10 mM EDTA (pH 7.4) to observe the headgroup PO₂⁻ stretching band. Initially, 3–4 mg of dry lipid was hydrated by the addition of 50 μ l of the appropriate buffer followed by vigorous vortexing at temperatures well above the T_m of the sample concerned. This dispersion was then squeezed between the CaF₂ windows of a heatable liquid cell (equipped with a teflon spacer) to form a 10- μ m film. Once the sample was mounted in the holder of the instrument, its temperature could be controlled (between -20° and 90°C) by an external, computer-controlled circulating water bath. The spectra were recorded with a Digilab FTS-40 instrument (Biorad Digilab Division, Cambridge, MA) using data acquisition and processing protocols described by Mantsch et al. (1985). The spectra obtained were analyzed with software supplied by Digilab and other computer programs obtained from the National Research Council of Canada, and plotted with Microcal Software. In the analysis of the contours of the C=O stretching 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, respectively) and band narrowing factors (1.8–2), as defined by the software package supplied by Digilab. 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 estimate 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

Differential scanning calorimetry

DSC thermograms illustrating the heating and cooling behavior of freshly dispersed samples of the *n*-saturated 1,2-diacyl PGs studied are illustrated in Fig. 1. When dispersed in media of physiologically relevant pH and ionic strength, unannealed samples of these lipids usually exhibit two structurally distinct thermotropic phase transitions, the transition temperatures of which progressively increase as the length of the hydrocarbon chain increases. The more prom-

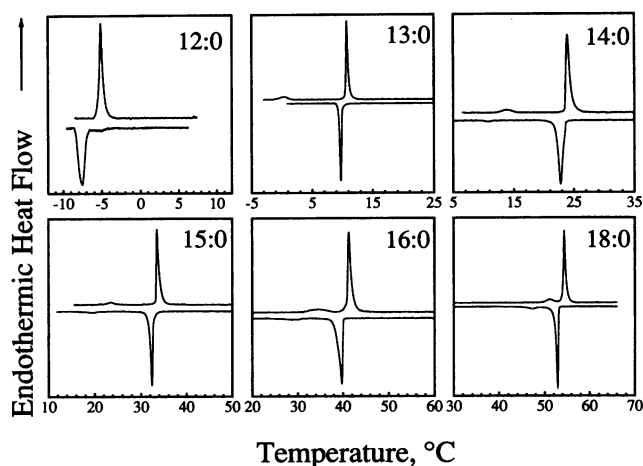


FIGURE 1 High-sensitivity heating and cooling DSC thermograms exhibited by the *n*-saturated 1,2-diacyl *sn*-glycero-3-phosphoryl-*rac*-glycerols. The thermograms shown were obtained samples dispersed in 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7.4) and recorded with the Hart instrument operating at scan rates near 15°C/h.

inent of these, the gel/liquid-crystalline phase transition, is fairly energetic and highly cooperative (see data listed in Table 1), and, as evidenced by the absence of significant cooling hysteresis (see Fig. 1), it is also freely reversible. These properties are similar to those of the lamellar gel/liquid-crystalline phase transitions of *n*-saturated 1,2-diacyl PCs (see Lewis et al., 1987a, and references cited therein), or the melting of the rotator phases of normal alkanes (see Broadhurst, 1962), in which a thermally induced onset of *trans-gauche* isomerism is nucleated from a solid-like phase in which all-*trans* hydrocarbon chains are orientationally

disordered (Doucet et al., 1981). Furthermore, as is typical of hydrocarbon chain-melting phase transitions, the gel/liquid-crystalline phase transition temperatures of these *n*-saturated 1,2-diacyl PGs increase smoothly but nonlinearly with increasing hydrocarbon chain length (see Fig. 3).

With the possible exception of 12:0 PG, unannealed samples of each of the lipids studied exhibit another weakly energetic phase transition at temperatures below that of the gel-liquid-crystalline phase transition described above. This lower-temperature transition is fairly broad and only weakly energetic (see data listed in Table 1) and exhibits a modest cooling hysteresis (see Fig. 1). From the data presented in Fig. 1 and in Table 1, it is evident that the midpoint temperature of this solid-state thermotropic event progressively increases as the hydrocarbon chain lengthens and that the temperature separation between this event and the gel/liquid-crystalline phase transition progressively decreases as the lengths of the PG hydrocarbon chains increase. These properties are very similar to those of the well-characterized pretransition exhibited by the *n*-saturated 1–2 diacyl PCs (for a full description of the calorimetric behavior of PC pretransitions, see Lewis et al., 1987a) and, by analogy, our observations support the conclusion that the structural events that occur during this PG solid-phase transition are probably similar to those of the so-called pretransition (i.e., the L'_β/P'_β phase transition; L_β is the lamellar gel phase, used later to describe both the L'_β and P'_β phases) of the *n*-saturated 1,2-diacyl PCs. In turn, this suggests that the structural events accompanying the gel/liquid-crystalline phase transitions of these PGs are, in effect, P'_β/L_α (lamellar liquid crystalline phase)-type chain-melting phase transitions. However, although our assignments are compatible

TABLE 1 Thermodynamic characterization of the phase transitions of the *n*-saturated 1,2-diacylphosphatidylglycerols

<i>N</i>	$L_c1^a-L'_\beta$	$L_c1^a-L_\alpha$	$L_c1^b-L'_\beta$	$L_c1^b-L_\alpha$	L_c2-L_α	$L'_\beta-P'_\beta$	$P'_\beta-L_\alpha$
I. Phase Transition Temperatures*							
				Temperature (°C)			
12	NO	NO	NO	13–16 [§]	26.4	ND [¶]	–5.1
13	NO	21.2	NO	NO	34.6	0.3	10.7
14	NO	NO	NO	24.5	40.3	13.7	23.9
15	11.4	NO	NO	NO	47.5	23.5	33.5
16	NO	NO	30.1	NO	45.7	34.6	41.3
18	NO	NO	37.1	NO	NO	51.1	54.4
II. Phase transition enthalpies*							
				Enthalpy (kcal/mol)			
12	NO	NO	NO	ND [¶]	12.3	ND	1.6
13	NO	ND [¶]	NO	NO	13.3	0.4	2.9
14	NO	NO	NO	ND [¶]	14.2	0.6	5.6
15	ND [¶]	NO	NO	NO	15.9	0.8	7.3
16	NO	NO	ND [¶]	NO	15.8	1.4	8.9
18	NO	NO	8.4	NO	NO	1.5	11.3

*Data obtained from DSC thermograms recorded in 50 mM Tris, 100 mM NaCl, 10 mM EDTA (pH 7.4).

[¶]Projected value of this parameter outside the range of our DSC instruments.

[§]Precise value unknown because of overlapping exothermic events.

[¶]Value unknown. Pure samples of the phase are too unstable to for calorimetric characterization.

N, Number of carbon atoms per hydrocarbon chain. NO, Not observed (phenomena not exhibited or not induced under our conditions). ND, Value not measured. L_c1^a , L_c1^b , Meta-stable quasicrystalline phases formed by PGs with odd- and even-numbered hydrocarbon chains, respectively (assigned as per FTIR spectroscopic data). L_c2 , Stable quasicrystalline phase (assigned per FTIR spectroscopic data).

with previously published studies of fully hydrated 1,2-dipalmitoyl *sn*-glycero-3-phosphoryl-*rac*-glycerol bilayers (see Watts et al., 1978), they should be regarded as tentative until more definitive structural studies such as x-ray diffraction are performed.

DSC thermograms that typify the behavior observed after short- and long-term regimes of low-temperature incubation of the *n*-saturated 1,2-diacyl PGs are illustrated in Fig. 2. It is clear that low-temperature annealing of these lipids dramatically alters their thermotropic phase behavior, as evidenced by the appearance of thermotropic events other than those described above. Our spectroscopic data confirm that the appearance of the latter is the result of the transformation of the L_{β} -type gel phase of these lipids into one or more highly ordered quasicrystalline polymorphs (see below). It is also clear that the pattern of thermotropic phase behavior observed after low-temperature annealing of fully hydrated samples of these lipids changes as the incubation period progresses to an extent that is hydrocarbon chain length dependent. For example, after a short (1–2 h) period of incubation at temperatures near -20°C , 12:0 PG exhibits a very complex pattern of behavior that often consists of highly energetic endothermic peaks centered between 10–16 $^{\circ}\text{C}$ and about 26 $^{\circ}\text{C}$. These thermograms may also contain markedly diminished endothermic events corresponding to the aforementioned P'_{β}/L_{α} phase transition as well as exothermic events between 10 $^{\circ}\text{C}$ and 20 $^{\circ}\text{C}$. Upon longer-term incubation ($\cong 12$ h), there is a gradual suppression of all other thermotropic events below 20 $^{\circ}\text{C}$, concomitant with the progressive growth in the intensity of a highly energetic peak near 26 $^{\circ}\text{C}$. The latter is the only significant thermotropic event observed after 10–12 h of incubation near

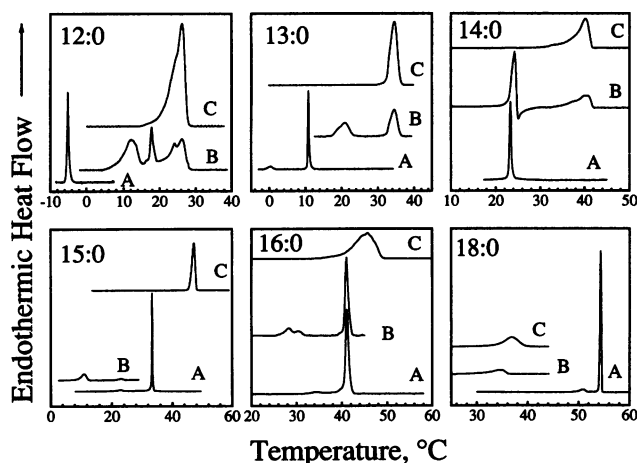


FIGURE 2 High-sensitivity DSC heating endotherms exhibited by the *n*-saturated 1,2-diacyl *sn*-glycero-3-phosphoryl-*rac*-glycerols. The thermograms shown were obtained samples dispersed in 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7.4) and acquired before low-temperature incubation (A), after short-term low-temperature incubation (B), and after extensive low-temperature incubation (C). With the exception of 12:0 and 13:0 PGs (Hart instrument), the thermograms shown were all recorded with the Microcal MC-2 high-sensitivity calorimeter.

-20°C . Further low-temperature annealing of 12:0 PG does not result in any significant changes in the general pattern of thermotropic phase behavior, although the remaining endothermic peak tends to get narrower and the measured peak temperatures and peak enthalpies tend to drift upward ($\leq 1^{\circ}\text{C}$ and ≤ 2 kcal/mol, respectively) as the length of the incubation period increases. These observations clearly suggest that the L_{β} -type gel phase of 12:0 PG is meta-stable with respect to at least two higher-melting polymorphs and thus readily transforms to such structures upon low-temperature incubation. Moreover, it is also clear that the polymorphic forms that give rise to the calorimetric signals detected between 10 $^{\circ}\text{C}$ and 20 $^{\circ}\text{C}$ are themselves meta-stable and that given enough time, they will convert to the form that gives rise to the endothermic peak near 26 $^{\circ}\text{C}$. Under our conditions the latter is the only structure that persists after 10–12 h of incubation at temperatures near -20°C , and appears to be the only structure that is thermodynamically stable under those conditions. For the most part, similar (but not identical) patterns of behavior are observed with most of the longer chain homologs, although the progression of events tends to occur on a progressively longer time scale as the lipid hydrocarbon chain length increases. For example, with 1,2-dimyristoyl *sn*-glycero-3-phosphoryl-*rac*-glycerol (DMPG), calorimetric evidence for the formation of “meta-stable intermediates” is first detected after 1–2 days of incubation at temperatures near 0–4 $^{\circ}\text{C}$, the stable, high-melting polymorph is calorimetrically detected after 6–8 days, and complete conversion to the latter occurs over a time scale of 2–3 weeks. In the case of 18:0 PG, there was no evidence for the formation of a stable high-melting polymorph over the time scale of this study ($\cong 2$ years). However, our FTIR data indicate that the quasicrystalline polymorphs that formed over this time scale exhibit spectroscopic signatures that closely resemble those of the “meta-stable intermediates” identified in our studies of the shorter chain homologs. It should be noted, however, that the chain length dependence alluded to above is not a continuous function of hydrocarbon chain length, because the kinetics of the process observed with PGs whose hydrocarbon chains contain an odd number of carbon atoms tend to be slower than is observed with their neighboring even-numbered homologs. We find that under comparable conditions, the L_{β} phases of the various PGs convert to quasicrystalline polymorphs at rates that can be ranked thus: 12:0 \gg 14:0 \cong 13:0 \gg 16:0 $>$ 15:0 \gg 18:0. We also find that with the probable exception of 18:0 PG, the formation of both the stable and meta-stable quasicrystalline polymorphs of these lipids can be accelerated by a brief (12:0 PG \cong 0.5–1 h; 14:0 PG \cong 12 h; 16:0 PG \cong 2 days) incubation at temperatures near -30°C , followed by a period of annealing at temperatures some 10–20 $^{\circ}\text{C}$ below the P'_{β}/L_{α} phase transition temperature. However, our time-resolved FTIR spectroscopic studies (data not shown) indicate that these crystal-like structures do not form when the samples are incubated at temperatures near -30°C , even if the incubation period is extended to several days or weeks.

However, exposure to such low temperatures appears to nucleate the formation of such structures and, upon subsequent warming to temperatures near the P'_β/L_α phase transition temperature, growth of these new phases can be quite rapid (12:0 PG \cong a few minutes; 14:0 PG \cong a few hours; 16:0 PG \cong 8–10 days). In these studies, however, this protocol was used primarily to expedite the formation of the thermodynamically stable quasicrystalline polymorphic forms of these lipids, because we found that the progression of events was difficult to control once initiated as outlined above. With the short-chain homologs (i.e., 12:0–14:0 PG), virtually complete conversion to the stable quasicrystalline form could be achieved with two cycles of “nucleation and growth incubations,” whereas with the longer chain analogs, this required several (4–6) such cycles, which involved longer periods of incubation.

The data presented in Table 1 and Figs. 2 and 3 also support two major generalizations. First, the polymorphs that form upon short-term low-temperature incubation decompose at lower temperatures than do those that formed upon extensive low-temperature incubation (see Fig. 2). As noted above, the former are meta-stable intermediates formed en route to more stable structures. Our FTIR spectroscopic studies (see below) indicate that the meta-stable intermediates and stable polymorphs are both crystal-like structures (i.e., quasicrystalline L_c (lamellar crystalline) phases) and that the structural features of the meta-stable intermediates (designated L_{c1}) are distinct from those of the stable polymorphs (designated L_{c2}) that are eventually formed. This conclusion is compatible with the results of recent DSC and electron microscopic studies of DMPG (Kodama et al., 1993). Furthermore, our spectroscopic data indicate that PGs whose hydrocarbon chains contain an odd number of carbon atoms form meta-stable crystal-like phases (designated L_{c1a}) that are spectroscopically distinct

from the meta-stable polymorphs (designated L_{c1b}) formed by the homologs whose hydrocarbon chains contain an even number of carbon atoms. The characteristics of the various L_c phases formed by these lipids will be examined in greater detail later. Second, the nature of the thermotropic phase transitions exhibited after periods of low-temperature annealing changes with increases in hydrocarbon chain length. For the shorter chain homologs such as 12:0 and 13:0 PG, seemingly direct conversions of their stable and meta-stable L_c phases to their respective liquid-crystalline states are observed at temperatures well above those of their P'_β/L_α phase transition temperatures, whereas with the longer-chain homologs, thermal decomposition of one or more of their quasicrystalline phases may be observed at temperatures below their P'_β/L_α phase transition temperatures. Moreover, at temperatures near the T_m of these lipids, we find that their quasicrystalline phases become progressively less stable with respect to their gel and liquid-crystalline phases as the length of the hydrocarbon chain increases. For the shorter chain lipids the L_β phase is meta-stable with respect to all of their quasicrystalline phases at all temperatures over which it is usually observed, and the liquid-crystalline phase is meta-stable with respect to one or more quasicrystalline structures at temperatures between the P'_β/L_α and L_c/L_α phase transition temperatures. This observation is also reflected by the fact that with increases in hydrocarbon chain length, the interval between their P'_β/L_α and L_c/L_α phase transition temperatures progressively diminishes (see Table 1 and Figs. 2 and 3). Moreover, an examination of the enthalpy changes involved in the thermal decomposition of the stable quasicrystalline phases of these lipids indicates that the difference between the total enthalpy of the L_c/L_α transition and the sum of the enthalpies of the pre- and gel/liquid-crystalline phase transitions decreases substantially as hydrocarbon chain length increases. The latter observation clearly suggests that the free energy of the stable quasicrystalline phases of these lipids decreases as the hydrocarbon chain length increases. Such phenomena have also been observed in studies of other phospho- and glycolipid bilayers (see Lewis et al., 1985a, 1987a,b; Mannock et al., 1988, 1990; and Lewis and McElhaney, 1993) and may well be a reflection of chain length-induced changes in the balance between the hydrophobic and hydrophilic contributions to the free energy of the L_c phase (see Discussion).

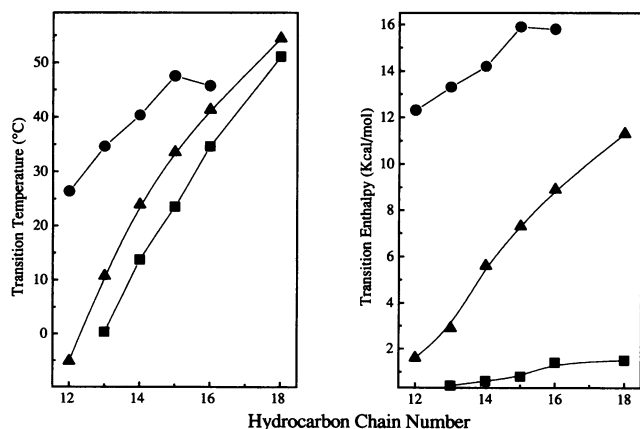


FIGURE 3 Chain length dependence of the transition temperatures (left) and transition enthalpies (right) of the n -saturated, 1,2-diacyl *sn*-glycero-3-phosphoryl-*rac*-glycerols. The temperatures and enthalpies were determined from DSC heating thermograms. \blacktriangle , P'_β/L_α (gel/liquid-crystalline) phase transitions. \blacksquare , L'_β/P'_β phase transitions (pretransitions). \bullet , $L_c/2L_\alpha$ phase transitions.

Fourier transform infrared spectroscopy

Thermotropic phase behavior

The left panel in Fig. 4 shows plots of the temperature dependence of the band maxima of the CH_2 symmetrical stretching bands of 14:0- and 18:0-PG and exemplifies various aspects of the phase behavior exhibited by the PGs before and after long periods of low-temperature incubation. The data show that discontinuous increases ($\cong 2\text{ cm}^{-1}$) in the frequency of the CH_2 symmetrical stretching band

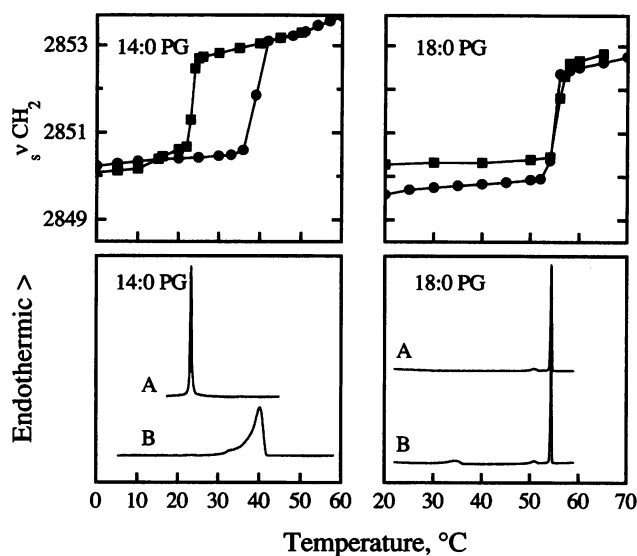


FIGURE 4 Temperature dependence of the frequency of the methylene symmetrical stretching bands exhibited by aqueous dispersions of 14:0 PG (top left) and 18:0-PG (top right). For each sample data were recorded in the heating mode before (A and ■) and after (B and ●) prolonged incubation at low temperature. To facilitate comparison of these data and the DSC results, the corresponding DSC heating thermograms are shown in the bottom panels. Samples were dispersed in D₂O buffered with 100 mM phosphate (pH 7.4).

occur at temperatures that coincide with either the typical gel/liquid-crystalline phase transition or the high-temperature thermal events observed after low-temperature incubation of these lipids. This particular spectroscopic change is also accompanied by a discontinuous increase in the width of the CH₂ symmetrical stretching band (data not shown). Together, these particular spectroscopic changes are indicative of the increase in conformational disorder (i.e., the onset of *trans-gauche* isomerism) that occurs when all-*trans* polymethylene chains melt and clearly identify these particular thermotropic events as hydrocarbon chain-melting phenomena (Snyder, 1967). It should be noted, however, that the spectroscopic changes described above are not observed at any of the thermotropic events that occur at temperatures below T_m . Therefore, the latter transitions are solid-phase events in which interconversions between various gel and/or quasicrystalline forms of these lipids occur.

Illustrated in Fig. 5 is an example of the spectroscopic changes that accompany the thermal decomposition of one of the L_c phases detected in our DSC studies. With the example shown (15:0 PG), DSC detects a single phase transition centered near 47°C (see Fig. 2), and in the corresponding FTIR spectroscopic experiment, changes in the C=O stretching and CH₂ scissoring bands (see Fig. 5), in the absorption bands arising from vibrations of the phosphate headgroup (data not shown), and in the CH₂ stretching bands (see previous paragraph) are observed over the same temperature range. At temperatures below 47°C absorption bands throughout the infrared spectrum are very sharp, and in many instances subcomponents of individual absorption

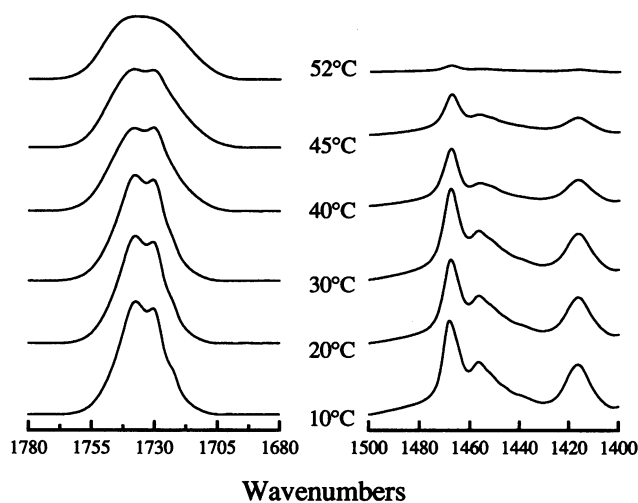


FIGURE 5 Temperature-dependent changes in the contours of the C=O stretching (left) and CH₂ scissoring (right) bands of 15:0 PG. The absorbance spectra shown were obtained from a sample that was extensively incubated at low temperatures. Spectra were recorded in the heating mode. The sample was dispersed in D₂O buffered with 100 mM phosphate (pH 7.4).

bands can be easily resolved. At temperatures above 47°C, the absorption bands broaden substantially, and decreases in the intensities of many of these bands are observed. These observations indicate that long-term low-temperature incubation of the sample results in the formation of a crystal-like phase in which infrared-active groups in both the polar and apolar domains of the lipid bilayer are essentially immobile (see Lewis and McElhane, 1996). The spectroscopic data also indicate that as the quasicrystalline structure breaks down, the mobility of all infrared-active groups increases, and that hydrocarbon chain conformational disorder increases when the sample is heated to temperatures above 47°C. These observations, coupled with evidence that the lamellar structure is maintained at high temperatures (see ³¹P-NMR spectroscopic data below), enable the unambiguous assignment of the thermotropic phase transition observed to a L_c/L_α phase transition. Comparable analyses of the spectroscopic changes that occur at all of the thermotropic phase transitions exhibited under our conditions enabled us to identify all of the thermotropic phase transitions exhibited by these compounds. The assignments of the various phases observed are listed in Table 1, and the types of thermotropic phase transitions exhibited by the various PGs used in this study are summarized in the block diagram shown in Fig. 6.

FTIR spectroscopic characterization of the quasicrystalline, gel, and liquid-crystalline phases

The patterns of infrared absorption exhibited by the polymorphic forms of these PGs contain valuable information about intermolecular interactions that occur in the headgroup, interfacial, and hydrophobic domains of those lipid

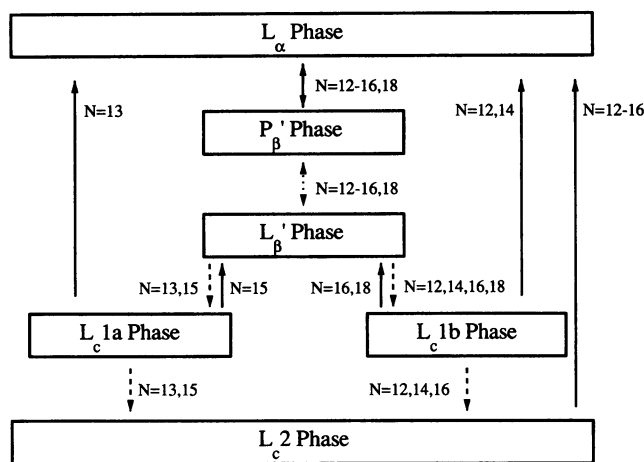


FIGURE 6 Block diagram summary of the pattern of polymorphic phase behavior exhibited by aqueous dispersions of the n -saturated 1,2-diacyl phosphatidylglycerols. N indicates the number of carbon atoms in the acyl chain; fast calorimetrically detectable processes are indicated by the solid arrows; slow calorimetrically detectable processes are indicated by the dotted arrows, and phase transitions that occur too slowly for calorimetric detection are indicated by the dashed arrows.

assemblies. For example, the CH_2 stretching region encodes information about the conformational disposition of the hydrocarbon chains (see previous section), whereas the $\text{C}=\text{O}$ stretching, CH_2 scissoring, and O-P-O asymmetrical stretching regions of the infrared spectrum each encode valuable information about lipid interfacial hydration/hydrogen bonding, lateral interactions between all-*trans* hydrocarbon chains, and phosphate headgroup hydration/hydrogen-bonding interactions, respectively (see Lewis and McElhaney, 1996). Such data can therefore provide valuable insight into the structures of the various phases observed, as well as the molecular basis of their behavior. The details and structural interpretation of our FTIR spectroscopic observations are presented below.

The $\text{C}=\text{O}$ stretching absorption bands

In the L_α phase the $\text{C}=\text{O}$ stretching bands are fairly broad, are centered near 1738 cm^{-1} , and seem to be a summation of subcomponents centered near 1741 and 1726 cm^{-1} (see Fig. 7). These component bands are also observed in the lamellar gel phases of these PGs and are probably attributable to differential infrared absorption by subpopulations of free and hydrogen-bonded ester carbonyl groups, respectively (see Blume et al., 1988; Lewis et al., 1994). Upon cooling, there tends to be an increase in the integrated intensity of the higher frequency component relative to that of the lower frequency component. This trend is more pronounced at the L_α/P'_β and P'_β/L'_β phase transitions and tends to be accentuated when the lamellar gel phase is cooled to very low temperatures. These observations imply that the structural events that occur at the conversion of the liquid-crystalline phase to the lamellar gel phase and upon

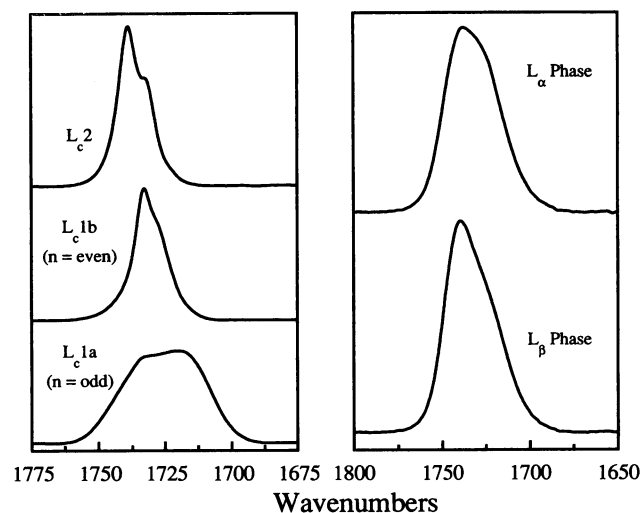


FIGURE 7 The $\text{C}=\text{O}$ stretching regions of the infrared spectra of the lamellar phases formed by the n -saturated 1,2-diacyl phosphatidylglycerols. Absorbance spectra are shown for the lamellar liquid-crystalline (L_α), the lamellar gel (L_β), and the lamellar crystalline phases formed upon short (L_{c1}) and long (L_{c2}) periods of incubation at low temperatures. The spectra of the lamellar crystalline phases were acquired at 0°C . Spectra of 14:0 PG were presented as exemplars of the even-numbered homologs, and of 15:0 PG as exemplars of the odd-numbered homologs. All data were obtained from samples dispersed in D_2O buffered with 100 mM phosphate (pH 7.4).

subsequent cooling of the latter are accompanied by a diminution of the population of hydrogen-bonded ester carbonyl groups. Most probably such occurrences are indicative of a general tendency toward decreased interfacial hydration upon cooling of both the gel and liquid-crystalline phases of these lipids.

Fig. 7 also indicates that the contours of the $\text{C}=\text{O}$ stretching bands of these PGs are drastically altered upon low-temperature incubation of these lipid dispersions. Under such conditions the lamellar gel phases initially convert to their meta-stable quasicrystalline polymorphs (see above), and the spectroscopic changes that accompany this conversion are strongly dependent upon whether the hydrocarbon chains contain an odd or even number of carbon atoms. With the odd-numbered compounds, the contours of the $\text{C}=\text{O}$ stretching band become strongly distorted toward the low-frequency end when the lamellar gel phase initially converts to the meta-stable crystalline polymorph (designated L_{c1a} ; see Fig. 7), because of the emergence of an additional low-frequency band near 1717 cm^{-1} (see Fig. 7, L_{c1a} phase). The population of ester carbonyl groups that gives rise to the latter band is probably more strongly hydrogen-bonded than normally observed in the gel and liquid-crystalline phases. Interestingly, our time-resolved studies of the emergence of this band also suggest that this new component band grows at the expense of the two components normally observed in the gel and liquid-crystalline phases of these lipids (data not shown). Given that an increase in the hydration of the bilayer polar/apolar interface seems unlikely under conditions favoring the formation

of crystalline polymorphs of these lipids, we suggest that this observation could be the result of the formation of crystal-like structures in which the ester carbonyl groups are strongly hydrogen bonded to either interfacial water or to the phosphoglyceryl headgroup. Interestingly, the contours of this particular C=O absorption band are very similar to those exhibited by the quasicrystalline phases formed by even-numbered isoacyl PCs (Mantsch et al., 1985), odd-numbered anteisoacyl PCs (Mantsch et al., 1987), and odd-numbered ω -cyclohexyl diacyl PCs (Mantsch et al., 1989).

In the case of the even-numbered compounds, low-temperature incubation of the lamellar gel phase initially results in the formation of a meta-stable quasicrystalline phase (designated L_c1b) that exhibits C=O stretching band contours markedly different from that formed by the odd-numbered compounds (see Fig. 7). Upon initial low-temperature incubation, there is a marked narrowing of the C=O absorption band and a drift of the band maximum from about 1739 cm^{-1} to values near 1732 cm^{-1} . Our analyses of the substructures of these absorption bands indicate that these observations are primarily the result of the disappearance of the high-frequency component normally observed in the gel and liquid-crystalline states ($\cong 1741\text{ cm}^{-1}$) and its apparent replacement by a narrower band centered near 1732 cm^{-1} (see Fig. 7). These changes indicate that the conversion to this particular meta-stable quasicrystalline phase results in a general reduction in the mobility of the ester carbonyl groups (note the marked narrowing of the C=O absorption band) and a marked change in either the pattern of interfacial hydrogen-bonding interactions and/or a net reduction in the polarity of the environment surrounding the ester carbonyl groups (note the net shift to lower absorption maxima). Because an increase in bilayer interfacial hydration seems unlikely under conditions favoring the formation of the crystalline polymorphs of these PGs, we suggest that the changes noted above are a reflection of conformational and/or orientational changes that increase the polarity of the environment surrounding one or more populations of ester carbonyl groups. It should also be noted that the spectroscopic observations described above are virtually identical to those reported in a previous FTIR spectroscopic study of DMPG (Epand et al., 1992). In that study, however, the authors seemed to be unaware that they were observing a meta-stable intermediate and assumed that they were observing the stable high-melting quasicrystalline phase.

Despite obvious spectroscopic differences between the meta-stable quasicrystalline phases of the odd- and even-numbered homologs, we find that upon prolonged low-temperature incubation the contours of the C=O stretching bands exhibited by both groups converge to a common form that is distinct from that exhibited by either of the meta-stable, quasicrystalline polymorphs described above. This form is characteristic of the stable quasicrystalline polymorph (designated L_c2) and consists of a narrow band centered near 1739 cm^{-1} , with a comparably narrow shoulder centered near 1732 cm^{-1} (see Fig. 7, L_c2 phase). When

compared with the C=O stretching bands of the two quasicrystalline polymorphs described above, it is clear that the conversion to the stable quasicrystalline polymorph is accompanied by a net drift of the C=O absorption bands to higher frequencies. This observation is consistent with the suggestion that final conversion to the stable crystal-like phase may involve further dehydration of the bilayer polar/apolar interface and/or the location of the ester carbonyl groups in less polar environments. Moreover, because these bands are also very narrow, we can also conclude that the ester carbonyl groups must be strongly immobilized.

The CH₂ scissoring absorption band

The contours of the CH₂ scissoring bands observed in the lamellar gel, the lamellar liquid-crystalline, and each of the quasicrystalline polymorphs identified are shown in Fig. 8. In the liquid-crystalline phase, the CH₂ scissoring band appears as a broad absorption band that is relatively weak and is centered near 1468 cm^{-1} . These features are indicative of a structure in which hydrocarbon chains are conformationally and orientationally disordered with very weak lateral interactions. In the L'_β and P'_β phases, the CH₂ scissoring band appears as a narrow, moderately intense band that is also centered near 1468 cm^{-1} . Although this band exhibits no discernible changes in frequency at the L'_β/P'_β phase transition, we generally find that if cooled to temperatures well below ($\geq 40^\circ\text{C}$) the pretransition temperature, a weak correlation field splitting of the CH₂ scissoring band begins to emerge. Under such conditions the CH₂ scissoring

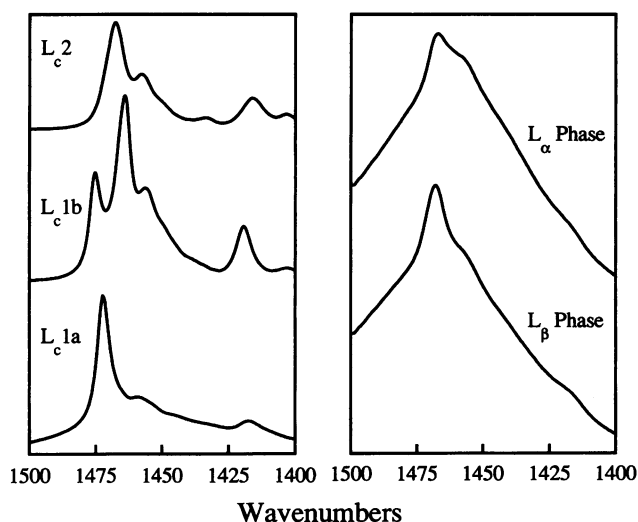


FIGURE 8 The CH₂ scissoring regions of the infrared spectra of the lamellar phases formed by the *n*-saturated 1,2-diacyl phosphatidylglycerols. Absorbance spectra are shown for lamellar liquid-crystalline (L_α), lamellar gel (L_β), and lamellar crystalline phases formed upon short (L_c1) and long (L_c2) periods of incubation at low temperatures. The spectra of the lamellar crystalline phases were acquired at 0°C . Spectra of 14:0 PG were presented as exemplars of the even-numbered homologs, and of 15:0 PG as exemplars of the odd-numbered homologs. All data were obtained from samples dispersed in D_2O buffered with 100 mM phosphate (pH 7.4).

band appears as a poorly resolved doublet whose components are initially centered near 1470 and 1466 cm^{-1} and tend to drift slowly apart upon further cooling (data not shown). Our observations therefore indicate that at temperatures relatively close to the hydrocarbon chain-melting phase transition temperature, the hydrocarbon chains of both lamellar gel phases retain a predominantly all-*trans* conformation in which lateral interchain interactions are relatively weak because the chains are orientationally disordered. These spectroscopic observations are also consistent with a structure in which the hydrocarbon chains are hexagonally packed and are undergoing either fast axial rotation or reorientational fluctuations of fairly large amplitude. The emergence of correlation field splitting of the CH_2 scissoring band at low temperatures also suggests that in the L'_β phase, the hydrocarbon chains tend to adopt orthorhombic perpendicular packing formats once rotational motions and other reorientational fluctuations are sufficiently damped by cooling.

An examination of the CH_2 scissoring bands shown in Fig. 8 provides further evidence that the meta-stable quasicrystalline phases formed by the odd- and even-numbered PG homologs are distinct from each other and from the stable quasicrystalline polymorph into which they are eventually transformed. In particular, the CH_2 scissoring band contours of the odd-numbered homologs consist of a single sharp band centered near 1472 cm^{-1} , whereas those of the corresponding even-numbered compounds consist of a well-resolved doublet with maxima centered near 1475 and 1464 cm^{-1} (see Fig. 8). From these data we can conclude that in the meta-stable quasicrystalline polymorphs of the odd- and even-numbered members of this homologous series, the subcellular packing formats adopted by the hydrocarbon chains are fundamentally different. For the odd-numbered compounds the appearance of a single CH_2 scissoring band near 1472 cm^{-1} is indicative of the formation of subcells in which the zigzag planes of the hydrocarbon chains are parallel to each other (probably a triclinic subcell). In this regard this PG subgel phase is also spectroscopically remarkably similar to the quasicrystalline phases of the even-numbered isoacyl PCs (Mantsch et al., 1985), the odd-numbered anteisoacyl PCs (Mantsch et al., 1987), and the odd-numbered ω -cyclohexyl diacyl PCs (Mantsch et al., 1989). With the even-numbered PGs, however, the appearance of the CH_2 scissoring band as a well-resolved doublet indicates that there is a strong correlation field splitting of this band (Snyder, 1961, 1979). In turn, the latter indicates that the lipid molecules are assembled with the zigzag planes of their hydrocarbon chains perpendicular to each other (probably an orthorhombic \perp subcell). This mode of subcellular packing differs markedly from that deduced from single-crystal x-ray studies of DMPG (see Pascher et al., 1987). However, these particular observations are also similar to those reported in a previous FTIR spectroscopic study in which it was erroneously assumed that the stable quasicrystalline phase was being observed (Epanand et al., 1992).

With both the odd- and even-numbered homologs, the CH_2 scissoring absorption band observed in the stable quasicrystalline phase consists of a single sharp band centered near 1468 cm^{-1} (see Fig. 8). The frequency of this band is similar to that observed in the lamellar gel phases described earlier and is consistent with the assembly of the lipid hydrocarbon chains into a global lattice of orientationally disordered all-*trans* polymethylene chains. Evidently the conversion of the meta-stable quasicrystalline phases of both the odd- and even-numbered homologs to the stable polymorph involves a significant rearrangement of the hydrocarbon chain subcellular packing formats that are observed in the meta-stable quasicrystalline phases. It should be noted, however, that unlike the lamellar gel phases described above, there are strong indications that the headgroup and interfacial moieties of the lipid molecules are strongly immobilized in the stable quasicrystalline phase (see above and ^{31}P -NMR spectroscopy section). Because immobilization of the headgroup and interfacial regions of these lipid molecules is incompatible with significant dynamic disorder in the hydrocarbon chains, one must conclude that the orientational disorder that occurs in the stable quasicrystalline phase must differ significantly from that which occurs in the lamellar gel phases described earlier. Most probably the all-*trans* hydrocarbon chains are arranged in a close-packed assembly in which they are essentially immobile (i.e., large-amplitude fluctuations have been suppressed), with their zigzag planes randomly oriented relative to each other. This conclusion also differs markedly from those drawn from single-crystal x-ray diffraction studies of DMPG (see Pascher et al., 1987).

The O-P-O asymmetrical stretching absorption band

The phosphate O-P-O asymmetrical stretching absorption bands of these PGs reveal a number of structurally interesting features. In the liquid-crystalline phase the contours of this absorption band are fairly broad and centered near 1215 cm^{-1} (see Fig. 9). In the lamellar gel phases this O-P-O absorption band remains broad, but its absorption maximum is observed at frequencies near 1205 cm^{-1} (see Fig. 9). The range covered by these absorption maxima is significantly lower than that normally observed with aqueous dispersions of the more common phospholipids (1220–1230 cm^{-1} ; see Wong and Mantsch, 1988; Ter-Minassian-Saraga et al., 1988; Hübner and Mantsch, 1991; Lewis et al., 1996, and references cited therein). The latter observation suggests that in the gel and liquid-crystalline phases of hydrated PG bilayers, phosphate moieties reside in more polar environments, are better hydrated, and/or are involved in stronger hydrogen-bonding interactions than is the case with either the gel and liquid-crystalline phases of other common phospholipids. In the quasicrystalline phases of these PGs, the contours of the O-P-O asymmetrical stretching band tend to be narrower in the gel and liquid-crystalline phases, al-

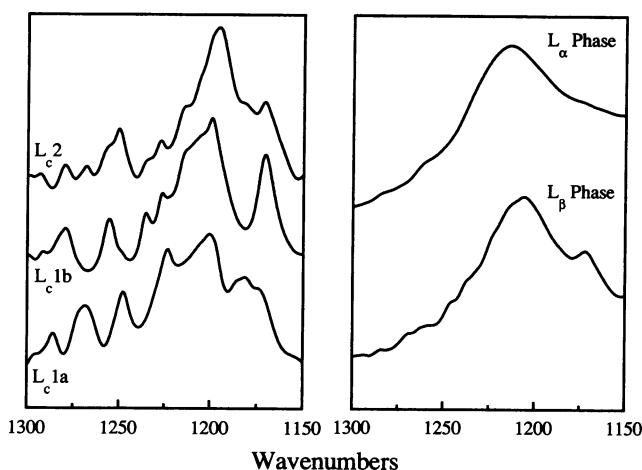


FIGURE 9 The asymmetrical phosphate stretching regions of the infrared spectra of the lamellar phases formed by the *n*-saturated 1,2-diacyl phosphatidylglycerols. Absorbance spectra are shown for lamellar liquid-crystalline (L_{α}), lamellar gel (L_{β}), and lamellar crystalline phases formed upon short (L_{c1}) and long (L_{c2}) periods of incubation at low temperatures. The spectra of the lamellar crystalline phases were acquired at 0°C. Spectra of 14:0 PG were presented as exemplars of the even-numbered homologs, and of 15:0 PG as exemplars of the odd-numbered homologs. All data were obtained from samples dispersed in H_2O with 50 mM Tris, 100 mM NaCl, and 10 mM EDTA (pH 7.4).

though their detailed contours tend to be obscured by several sharp bands arising from the CH_2 wagging band progression (see Fig. 9). However, a cursory examination of the underlying band suggests that the band maxima remain near 1205 cm^{-1} in the two types of meta-stable quasicrystalline phases observed, but decrease to values near 1195 cm^{-1} when these meta-stable phases convert to the stable quasicrystalline polymorph (see Fig. 9). These observations suggest that upon conversion to the meta-stable quasicrystalline phases, the mobility of the phosphate groups decreases significantly (note the narrowing of the absorption band), whereas the overall polarity of its environment remains comparable to that observed in the lamellar gel phases. Upon conversion to the stable quasicrystalline phase, however, there seems to be either an increase in the strength of hydrogen-bonding interactions involving the phosphate moiety and/or a further increase in the polarity of its environment.

Finally, another interesting feature of the region of the infrared spectrum that encompasses the O-P-O asymmetrical stretching absorption bands is the observation that absorption bands arising from the CH_2 wagging band progression are relatively weak in the gel phase (see Fig. 9). In fact we actually find that these bands tend to remain relatively weak until the samples are cooled to temperatures well below ($\geq 40^\circ\text{C}$) the T_m (data not shown). This situation differs markedly from that observed with most of the other lipid bilayers examined so far (for examples see Chia et al., 1993; Moore et al., 1993; Lewis et al., 1996). These observations suggest that the all-*trans* hydrocarbon chains in the lamellar gel phases of these PG bilayers may be more

loosely organized than is the case with many other types of lipid bilayers, and imply that at temperatures relatively close to T_m , the amplitude of torsional motions in the all-*trans* polymethylene chains are great enough to result in a marked diminution in the intensity of the CH_2 wagging band progression bands. However, because the intensity of these bands increases markedly upon cooling to temperatures well below T_m and upon conversion to either the meta-stable or stable quasicrystalline phases (see Fig. 9), it is evident that the amplitude of such motions decreases significantly upon cooling and when the lamellar gel phase converts to either of the quasicrystalline polymorphs. It is also possible that the marked increase in the intensity of the CH_2 wagging bands coincident with the formation of the quasicrystalline polymorphs could be the result of a conformational change in the glycerol moieties of these lipids.

^{31}P nuclear magnetic resonance spectroscopy

^{31}P -NMR powder patterns that typify those exhibited by the PGs studied are presented in Fig. 10. Such data are sensitive to the motional properties of the phosphate headgroup and can provide valuable information about the nature of the phospholipid macromolecular assembly observed (see Seelig, 1978a). Spectrum B in Fig. 10 describes an axially symmetrical powder pattern that typifies the L_{β} -type gel phases of normal phospholipid bilayers as well as the L'_{β}

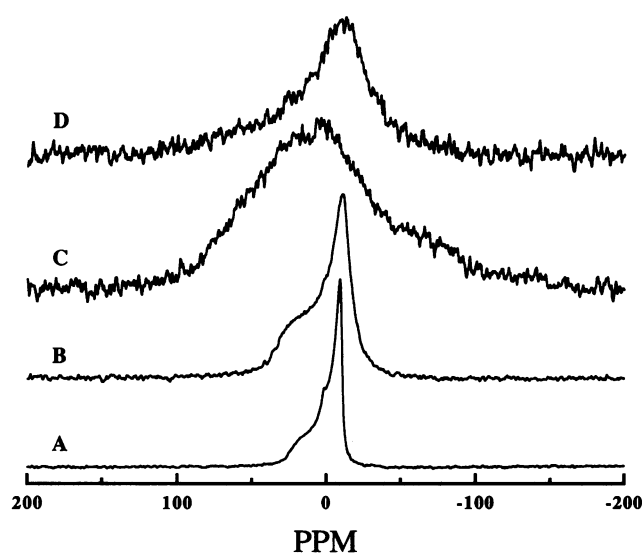


FIGURE 10 Proton decoupled ^{31}P -NMR spectra exhibited by the lamellar phases of the *n*-saturated 1,2-diacyl phosphatidylglycerols. The data shown are typical of (A) the liquid-crystalline phase (L_{α}); (B) the lamellar gel phases (L_{β}); (C) the L_{c1} lamellar crystalline phases (short-term low-temperature incubation); and (D) the L_{c2} lamellar crystalline phase (long-term low-temperature incubation). Spectra shown were acquired with the same sample (DMPG) and involved the processing of 2000 transients for spectra A and B, 12,000 transients for spectrum C, and 60,000 transients for spectrum D. Spectra were acquired with lipids dispersed in a buffer containing 50 mM Tris, 100 mM NaCl, 10 mM EDTA (pH 7.4). The spectra shown were acquired at 35°C (A), 15°C (B), 0°C (C), and 0°C (D).

and P'_β phases of the PGs used in this study. At low temperatures (i.e., in the L'_β phase), the basal linewidths of these powder patterns approach 80–85 ppm. These values decrease continuously upon heating and fall to values near 70–75 ppm upon conversion to the P'_β phase. Such changes reflect the thermally induced increases in the mobility of the phosphate headgroup that occur when the L'_β phase is heated and eventually converts to the P'_β phase. Comparable thermally induced decreases in basal linewidth also occur in the P'_β phase. However, at the P'_β/L_α phase transition, a discontinuous decrease in basal line occurs and powder patterns with basal linewidths near 45 ppm are observed (see Fig. 10, spectrum A). Such spectra are consistent with the existence of liquid-crystalline lamellar phases in which the phosphate headgroups are undergoing fast, axially symmetrical motion and have been observed in the L_α phases of all of the common phospholipids examined so far.

The powder patterns observed after low-temperature incubation of these lipids differ radically from those exhibited by their L'_β and P'_β phases. The spectrum C in Fig. 10 describes a very broad powder pattern (basal line width $\cong 220$ –230 ppm), which typifies that observed upon short-term incubation of these PGs (i.e., conditions favoring the formation of the meta-stable L_{c1} crystal-like phases of both the odd- and even-numbered homologs). Furthermore, the basal linewidths approach those previously observed in the quasicrystalline phases exhibited by some mixed-chain PCs (for examples see Lewis et al., 1984) and dried phosphatidylcholine powders (see Seelig, 1978). These observations indicate that the meta-stable L_{c1} phases are well-ordered structures in which axial reorientation of the phosphate headgroups occurs very slowly on the ^{31}P -NMR time scale.

Spectrum D in Fig. 10 typifies that obtained in our attempts at ^{31}P -NMR spectroscopic examination of the stable quasicrystalline phases (i.e., the L_{c2} phases) of these lipids. The spectra observed are dominated by resonances about 13 ppm upfield, a feature that is atypical of the crystal-like phases of all common phospholipids examined so far. We also found that conversion to the L_{c2} phase results in a very drastic decline in our signal intensities, and despite the acquisition of a very large number of transients (for an example, see legend to Fig. 10), very poor spectra were obtained. Interestingly, similar problems have been encountered previously when attempts have been made to record ^{31}P -NMR spectra of the stable crystal-like phases of PE bilayers (Xu et al., 1988; Lewis and McElhaney, 1993). Such problems are believed to be the result of the marked reduction in the transverse relaxation rates of the ^{31}P nucleus that usually occurs when phosphate groups become locked in long-lived, strongly hydrogen-bonded structures (see Withers et al., 1985). It should also be noted that the peak intensity observed about 13 ppm upfield is more typical of phospholipid L_β and L_α phases in which axial reorientation of the phosphate headgroup occurs at rates that are comparable to the ^{31}P -NMR time scale. This observation, coupled with the drastic loss of spectral intensity coincident with the formation of the L_{c2} phase and the fact

that transformation of the L_β phases of these lipids to the stable crystal-like phases tends to be very slow, suggests the possibility that the bulk of the observed spectral intensity shown in Fig. 10 D actually arises from small residual domains of the L_β phase that have been trapped among domains of the L_{c2} phase. If such is the case, then our inability to adequately record the ^{31}P -NMR spectrum of the stable crystal-like phases of these PGs may itself be a reflection of the formation of structures in which the phosphate headgroups have been effectively locked in stable, long-lived hydrogen-bonding networks. The involvement of the PG phosphate in stable hydrogen bonding interactions with the headgroup glycerol hydroxyl groups has been noted in single-crystal x-ray diffraction studies of DMPG (Pascher et al., 1987).

DISCUSSION

These studies present some new data which, when combined with previously published work, can provide the basis of a better understanding of the properties of PG bilayers in general and of the complex polymorphic phase behavior exhibited by *n*-saturated 1,2-diacyl PGs. This work and previously published studies clearly show that under physiologically relevant conditions, the pattern of thermotropic phase behavior exhibited by freshly dispersed samples of these PGs is remarkably similar to that of the corresponding 1,2-diacyl PCs. In fact, with the probable exception of the enthalpy changes accompanying the hydrocarbon chain-melting phase transition, all of the primary thermodynamic parameters characteristic of this aspect of the phase behavior of PGs are of a magnitude comparable to those of the corresponding PCs. Thus, despite the fact that charged group interactions at the surfaces of PC and PG bilayers are bound to be markedly different, the structural transformations occurring at their prephase and main phase transitions appear to be very similar. Although similar observations have been noted elsewhere (for examples, see Watts et al., 1978; Watts and Marsh, 1981; Findlay and Barton, 1978), the reasons why this aspect of the behavior of PG bilayers should so closely resemble PC bilayers are not fully understood. With both PC and PG bilayers the net effects of steric crowding of their polar headgroups and charge repulsion between the phosphate moieties should reduce bilayer cohesion. However, intermolecular coulombic attraction between the quaternary nitrogen and the negatively charged phosphate groups of PC bilayers will partially compensate for these repulsive forces (see Seelig et al., 1987; MacDonald et al., 1991). With PG bilayers, however, there is no capacity for coulombic attraction, but there is considerable potential for intermolecular hydrogen bonding between the glycerol hydroxyl groups and the phosphate moiety of the polar headgroup (see Boggs, 1980, 1986, 1987; Pascher et al., 1987; Cevc, 1990; and references cited therein), and their headgroup hydroxyl groups may also partially mimic the solvation properties of water. Thus the aforementioned

similarities between PC and PG bilayers may occur because the net effects of intermolecular hydrogen bonding and hydration-like interactions between the phosphate and headgroup glyceryl hydroxyl groups at the surfaces of PG bilayers are comparable in magnitude to those of the intermolecular coulombic attractions occurring at the surfaces of PC bilayers. The suggestion of a specialized pattern of interactions involving the phosphate groups of PG bilayers is compatible with the results of previously published single-crystal x-ray diffraction studies (Pascher et al., 1987), and with our FTIR spectroscopic evidence that the environment around the phosphate moieties of PG bilayers differs markedly from that observed with the zwitterionic phospholipids examined so far.

The involvement of PG headgroup hydroxyls in intermolecular hydrogen bonding and hydration-like interactions can also explain the relative ease with which these lipids form condensed, partially dehydrated, quasicrystalline bilayers when incubated at low temperatures. Indeed, such interactions may be a prerequisite for the formation of the quasicrystalline phases of these lipids, because it is difficult to envisage how any extended array of tightly packed PG molecules can be assembled without some screening of their negative charges. The coordination of the PG headgroup hydroxyls to the phosphate moiety of an adjacent PG molecule would effect a partial screening of the phosphate

negative charge. Moreover, because such interactions will displace some solvation water from the phosphate moiety, such dehydration may well be an essential part of the nucleation and growth of these quasicrystalline structures. The process of displacing phosphate solvation water may also be made more kinetically and thermodynamically favorable by a decrease in the chemical activity of water such as occurs upon cooling to low temperatures.

Our observations have also presented a better picture of the structures of the polymorphic forms that these lipids adopt (see summary in Table 2) and revealed a number of specific and general features about the overall pattern of polymorphic phase behavior (see summary in Fig. 6). First, incubation of these compounds at low temperatures results in the formation of one or more quasicrystalline structures at rates that decrease with increases in hydrocarbon chain length. Moreover, the rates of formation of the L_c phases of homologs whose hydrocarbon chains contain an odd number of hydrocarbon atoms tend to be slower than what is observed with the neighboring even-numbered homologs. This overall pattern has been observed in comparable studies of other homologous series of phospho- and glycolipids (for examples see Lewis and McElhaney, 1985a,b, 1993; Lewis et al., 1987a; Mannock et al., 1988, 1990) and may well be a general property of lipid bilayers. Second, the meta-stable quasicrystalline phases of these compounds ex-

TABLE 2 Characterization of the C=O stretching, CH₂ scissoring, and O-P-O asymmetrical stretching bands of the *n*-saturated 1,2-diacyl phosphatidylglycerols

Lipid phase	C=O stretching (cm ⁻¹)*	CH ₂ scissoring (cm ⁻¹)*	O-P-O asym. stretching (cm ⁻¹)*	Comments
L _α	1738 (1741,1728)	1468	1214	Orientationally and conformationally disordered chains; mobile, well-hydrated interfacial groups; mobile, well-hydrated/strongly H-bonded phosphate
L _β [#]	1740 (1742,1729)	1468	1205	Orientationally disordered all- <i>trans</i> chains; interfacial groups less mobile & less hydrated than L _α phase; phosphate better hydrated and/or more strongly H-bonded than the L _α phase
L _c 1a	1723 (1741,1727,1716)	1472	1205	Triclinically packed all- <i>trans</i> chains; interfacial groups, well-hydrated and fairly immobile, large population of strongly H-bonded C=O groups; phosphate hydration/H-bonding comparable to L _β phase but phosphate groups are less mobile
L _c 1b	1732 (1732,1722)	1475,1464	1205	Orthorhombically packed all- <i>trans</i> chains; interfacial groups partially hydrated and fairly immobile; weaker patterns of interfacial H-bonding; smaller population of H-bonded C=O groups than L _c 1a phase; phosphate hydration/H-bonding comparable to L _β phase but phosphate groups are less mobile
L _c 2	1738 (1738,1732,1722)	1467	1195	Orientationally disordered all- <i>trans</i> chains; fairly dehydrated polar/apolar interface with small populations of H-bonded C=O groups; strongly immobilized, strongly H-bonded phosphate

*Values rounded to the nearest wavenumber.

[#]Refers to both the L_β and P_β phases.

hibit a distinct odd/even structural dimorphism (see Table 2 for a summary of the structural features of the meta-stable quasicrystalline phases). Odd/even structural dimorphism has also been observed in studies of PCs with branched and ω -cyclohexyl fatty acyl chains (Lewis and McElhaney, 1985a,b; Lewis et al., 1987b, 1989; Mantsch et al., 1985, 1987, 1989; Church et al., 1986) and appears to be a general property of long-chain paraffinic compounds whose acyl chains are tilted (Malkin, 1952; Broadhurst, 1962). We therefore suggest that in the meta-stable quasicrystalline phases of these PGs, the hydrocarbon chains of at least one of the two groups of homologs (i.e., the odd-numbered, even-numbered, or both) must be tilted to the bilayer normal. Interestingly, our FTIR spectroscopic data also suggest that in the quasicrystalline phases formed by the odd-numbered homologs, the hydrocarbon chains adopt a triclinic parallel subcellular packing format, whereas with the even-numbered counterparts, the hydrocarbon chains adopt an orthorhombic perpendicular packing format (see Table 2). It is generally observed that hydrocarbon chains that adopt the triclinic parallel subcellular packing format tend to be tilted, whereas those with orthorhombic perpendicular packing formats tend to be normal to the end-group planes (see Kitaigorodskii, 1961).

Despite the odd/even structural dimorphism observed in the meta-stable quasicrystalline intermediates, the stable quasicrystalline polymorphic forms of these lipids form an isostructural series with no evidence of structural polymorphism. This particular aspect of the behavior of these PGs differs markedly from that of the 1,2-diacyl PCs (see Lewis and McElhaney, 1990, 1992), but exhibits some similarities to corresponding PEs and α -D-glucopyranosyl-*sn*-glycerols (Lewis and McElhaney, 1993; Sen et al., 1990). These observations provide valuable insight into the nature of the forces that stabilize the stable quasicrystalline phases of these PGs. With PC bilayers one also observes a very distinctive pattern of chain length-dependent structural polymorphism (see Lewis and McElhaney, 1990). Such observations can be rationalized by suggesting that the geometric requirements for optimization of hydrophobic interactions between lipid hydrocarbon chains and of polar interactions in the headgroup and bilayer polar/apolar interface cannot be simultaneously met and that the magnitudes of the hydrophobic and polar contributions to the free energy of their quasicrystalline phases are of comparable magnitude. The observed chain length-dependent structural polymorphism will therefore reflect the progressive chain length-dependent changes in the balance between the hydrophobic and hydrophilic contributions to the free energy of the quasicrystalline phase. With the PEs and α -D-glucopyranosyl-*sn*-glycerols, there are no indications of chain length-dependent structural polymorphism, and there are clear indications that strong lateral interactions between the hydrocarbon chains are maintained at all hydrocarbon chain lengths examined (Lewis and McElhaney, 1993 and other unpublished data from this laboratory). With these latter lipids there may be thermodynamically favorable and kinet-

ically accessible conformations in which optimal or near-optimal interactions in the hydrophobic, hydrophilic, and interfacial domains of the lipid bilayer can be simultaneously maintained. With the PG bilayers, however, our spectroscopic data indicate that concomitant with the formation of the stable quasicrystalline phase is the evolution of strong hydrogen bonding interactions involving the phosphate moiety and a significant disruption of the hydrocarbon chain subcellular packing that had emerged in the meta-stable quasicrystalline phase (see Table 2). This observation clearly suggests that polar forces in the headgroup region are the predominant driving forces behind the formation of the stable quasicrystalline phases of these lipids, and implies the absence of thermodynamically favorable and/or kinetically accessible lipid conformations that permit the simultaneous maintenance of optimal intermolecular interactions in all regions of these PG bilayers. A clearer picture of the nature of the intermolecular interactions involved should emerge from x-ray diffraction, FTIR, and NMR spectroscopic studies of macroscopically oriented quasicrystalline samples of these lipids.

In conclusion, we suggest that the capacity for the PG polar headgroups to mimic some of the solvation properties of water may well be a distinctive and critical feature of the role that PG plays in biological membranes. Of the common naturally occurring phospholipids, PG, PI, and DPG are the only ones capable of competing with water for solvation and/or hydration sites at the surfaces of lipid bilayers (note that they all possess free headgroup hydroxyl groups). However, because of the restraints upon the conformational freedom of the hydroxyl groups of PI and DPG (the hydroxyl groups of PI are attached to a rigid ring system and the hydroxyl group of DPG is attached to the backbone of the hydrophilic spacer between the two hydrophobic domains of the lipid), their capacity to compete with water for those sites may be severely impaired. With PG, however, there are fewer restraints on the conformational freedom of the headgroup hydroxyls (the two hydroxyl groups are located at the end of a flexible chain), and as a result, they should be more effective competitors for hydration, hydrogen bonding, and/or solvation sites. We therefore suggest that this particular property may be the distinctive feature of PG molecules that enables them to confer upon lipid bilayers fairly specialized properties in addition to those attributable to the negative charge. Most probably such properties will be specific to PG-containing membranes and can therefore constitute the basis of some of the specialized roles that PG seems to perform in some cell membranes (for an example, see Xia and Dowhan, 1995b). We also note that PG is by far the dominant anionic membrane lipid found in procaryotic organisms, whose membranes often contain large amounts of neutral glycolipids. It would be very interesting to determine whether hydration-like interactions between phosphoglycerol and glycosyl moieties at the surfaces of those membranes are structurally and/or functionally important.

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