Studies of the Thermotropic Phase Behavior of Phosphatidylcholines Containing 2-Alkyl Substituted Fatty Acyl Chains: A New Class of Phosphatidylcholines Forming Inverted Nonlamellar Phases

Ruthven N. A. H. Lewis,* Ronald N. McElhaney,* Paul E. Harper,‡ David C. Turner,‡ and Sol M. Gruner‡
*Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7; and ‡Department of Physics, Princeton University, Princeton, New Jersey, 08544 USA

ABSTRACT We have synthesized a number of 1,2-diacyl phosphatidylcholines with hydrophobic substituents adjacent to the carbonyl group of the fatty acyl chain and studied their thermotropic phase behavior by differential scanning calorimetry, ^{31}P-nuclear magnetic resonance spectroscopy, and x-ray diffraction. Our results indicate that the hydrocarbon chain-melting phase transition temperatures of these lipids are lower than those of the n-saturated diacylphosphatidylcholines of similar chain length. In the gel phase, the 2-alkyl substituents on the fatty acyl chains seem to inhibit the formation of tightly packed, partially dehydrated, quasicrystalline bilayers (L_c phases), although possibly promoting the formation of chain-interdigitated bilayers. In the liquid-crystalline state, however, these 2-alkyl substituents destabilize the lamellar phase with respect to one or more inverted nonlamellar structures. In general, increases in the length, bulk, or rigidity of the alkyl substituent result in an increased destabilization of the lamellar gel and liquid-crystalline phases and a greater tendency to form inverted nonlamellar phases, the nature of which depends upon the size of the 2-alkyl substituent. Unlike normal non-lamella-forming lipids such as the phosphatidylethanolamines, increases in the length of the main acyl chain stabilize the lamellar phases and reduce the tendency to form nonlamellar structures. Our results establish that with a judicious choice of a 2-alkyl substituent and hydrocarbon chain length, phosphatidylcholines (and probably most other so-called "bilayer-preferring" lipids) can be induced to form a range of inverted nonlamellar structures at relatively low temperatures. The ability to vary the lamellar/nonlamellar phase preference of such lipids should be useful in studies of bilayer/nonbilayer phase transitions and of the molecular organization of various nonlamellar phases. Moreover, because the nonlamellar phases can easily be induced at physiologically relevant temperatures and hydration levels while avoiding changes in polar headgroup composition, this new class of 2-alkyl-substituted phosphatidylcholines should prove valuable in studies of the physiological role of non-lamella-forming lipids in reconstituted lipid-protein model membranes.

INTRODUCTION

The total membrane lipids of virtually all biological membranes studied to date form lamellar (bilayer) phases when dispersed in excess water under physiologically relevant conditions (Singer and Nicholson, 1972; McElhaney, 1984, 1989). However, almost all biological membranes contain nontrivial amounts of at least one lipid component which, in isolation, forms inverted nonlamellar (cubic or hexagonal) phases (Cullis et al., 1983; Rilfors et al., 1984; Gruner, 1985). In fact, in some microorganisms such as *Escherichia coli* (see Raetz, 1982 and references cited therein) or *Acholeplasma laidlawii* B enriched in particular types of fatty acids (Silvius et al., 1980; Bhakoo et al., 1987; Lewis et al., 1990); the so-called non-lamella-forming lipids predomimate. Moreover, experiments with microorganisms such as *Acholeplasma laidlawii* A (Wieslander et al., 1980, 1981; Rilfors et al., 1984; Lindblom et al., 1986) and *Clostridium butyricum* (Johnston and Goldfine, 1985; Goldfine et al., 1987a, b) indicate that the ratio of non-lamella- forming to bilayer-forming lipids is biosynthetically regulated so as to maintain a stable bilayer which nevertheless has some potential to form nonlamellar phases. Such findings suggest that non-lamella-forming lipids perform some important function (or functions) in biological membranes, but, despite considerable research effort and speculation, this function (or functions) remains unclear at present (Gruner, 1989).

Currently, there are two general views regarding the potential functions of non-lamella-forming lipids in biological membranes. One view is that these lipids actually induce the formation of transient, localized nonlamellar structures in the membrane under certain conditions. Such suggestions have been supported by studies of model lipid membranes (Verkleij et al., 1979; Ellens et al., 1989; Siegel et al., 1989; Siegel, 1986a, b) in which nonlamellar structures have been detected as possible fusion intermediates and where the presence of non-lamella-forming lipids seems to be necessary for efficient fusion under physiologically relevant conditions. It should be noted, however, that nonlamellar lipid structures are difficult to observe in biological membranes under physiologically relevant conditions. Moreover, the large scale formation of such structures seems unlikely in cell membranes

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Address reprint requests to Ronald N. McElhaney, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

David C. Turner's present address is Naval Research Laboratory, Code 6090, Washington, DC 20375 USA.

Abbreviations used in this paper: PE, phosphatidylethanolamine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; H_{II}, inverted hexagonal phase; L_α, lamellar liquid-crystalline phase; L_β, lamellar gel phase; T_m, gel/liquid-crystalline phase transition temperature.

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such as the plasma membrane, because the presence of any long lived nonbilayer structures will inevitably compromise the barrier properties of such membranes. Another view is that non-lamella-forming lipids impart to the cell membrane special properties that are essential for normal functioning (Gruner, 1985, 1989; Hui, 1987, Tate et al., 1991). For example, Gruner (1985, 1989) suggested that the curvature strain imparted by a non-lamella-forming lipid may modulate the activity of some membrane proteins. This suggestion is supported by observations of correlations between the biological activities of reconstituted membrane proteins and peptides and the content of a non-lamella-preferring lipid in the membrane (Navarro et al., 1984; Jensen and Schutzback, 1988; Wiedman et al., 1988; Keller et al., 1993). Also, the presence of lipids capable of forming nonlamellar structures seems to be essential for the optimal activity of certain membrane-bound enzymes and transport proteins (for a discussion, see Hui and Sen, 1989). However, alternative interpretations are possible mainly because it is difficult to unequivocally assay and demonstrate a fundamental relationship between membrane protein activity and the phase preference of lipids.

There have been a number of studies on lamellar/nonlamellar phase transitions and on the organization of the lipid molecules in various nonlamellar phases (for reviews, see Mariani et al., 1988; Lindblom and Rilfors, 1989; Seddon et al., 1990). Moreover, the molecular properties that predispose a lipid molecule to form nonlamellar phases (e.g., the effects of polar headgroup size and hydrocarbon chain length and volume on lamellar/nonlamellar phase behavior) can be rationalized by considerations of lipid "shape" (Israelachvilli et al., 1977, 1980) and/or lipid monolayer spontaneous curvature (Kirk et al., 1984; Gruner et al., 1985; Gruner, 1989). However, progress in our overall understanding of the molecular basis of lipid bilayer/non-bilayer phase behavior is limited by the fact that experimental work in this area of lipid research has traditionally involved the study of relatively few lipid species (mainly the diacyl and dialkyl PEs and a few monoglycosyldiacylglycerols). Studies of such a limited range of lipid molecules, no matter how detailed, can be limiting especially when attempts are made to formulate a broader description of the phenomena observed. For example, it is well known that the L_α/H_{II} phase transition temperatures of aqueous PE dispersions are significantly lowered by the introduction of *cis*-double bonds into the hydrocarbon chains, presumably because the *cis*-double bond increases the cross-sectional area occupied by the hydrocarbon chains relative to that of polar headgroup. However, a recent study of a large number of diacyl PEs demonstrated that for a given effective chain length, the L_α/H_{II} transition temperature occurs at a relatively constant reduced temperature above that of the L_β/L_α transition, irrespective of the chemical structure of the acyl chain (Lewis et al., 1989). Evidently, the presence of a *cis*-double bond lowers the L_α/H_{II} phase transition temperature because it lowers the L_β/L_α phase transition temperature as well and not because of a change in the shape of the PE molecule in the relevant L_α phase. The above discussion provides an example of how easily one can be misled when attempts are made to develop general principles on the basis of relatively few observations on a fairly small range of compounds. Given this, we have embarked upon a program designed to broaden the base of our knowledge of both naturally occurring and synthetic non-lamella-forming lipids by a careful study of the structural parameters that affect their predisposition to assemble into nonlamellar phases. We report here on a novel approach that involves structural modifications that induce the normally lamella-preferring diacyl PCs to form nonlamellar structures. In this study we explicitly concentrate on structural modifications of the fatty acyl chains of the lipid molecules to minimize potential changes in the essential character of headgroup and polar/apolar interfacial regions of the PC bilayer.

MATERIALS AND METHODS

The commercially available reagents used in this study were of at least analytical grade and were obtained from either Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO). For those diethyl alkyl malonates that were not commercially available, their syntheses were achieved as outlined below for diethyl cyclohexylmethyl malonate. 120 mmols of clean sodium were dissolved in 500 ml of anhydrous ethanol and 100 mmols of diethyl malonate were added. The mixture was then cooled in an ice bath and 100 mmols of cyclohexylmethyl bromide were slowly added with stirring. The mixture was next stirred at room temperature for 1 h, during which time the initiation of the reaction was indicated by the slow precipitation of sodium bromide. The mixture was then slowly warmed and finally refluxed overnight to ensure completion of the reaction. After cooling, the reaction mixture was quenched by pouring into 1 liter of cold dilute hydrochloric acid and subsequently extracted with hexane. The organic extract was then washed twice with water and once with saturated sodium chloride and then was dried over anhydrous sodium sulfate. After removal of the solvent by rotary evaporation, the crude diethyl cyclohexylmethyl malonate was purified by fractional distillation.

The diethyl alkyl malonates were converted to the (*dl*)-2-alkyl- substituted fatty acid precursors for the synthesis of the PCs used in this study using the procedure outlined below for the synthesis of (*dl*)-2-butyl hexadecanoic acid. 25 mmols of diethyl butyl malonate were converted to diethyl-(2-butyl, 2-myristyl)-malonate by reacting it with myristyl bromide under reaction conditions similar to that described above for the synthesis of diethyl cyclohexylmethyl malonate. After purification of the di-ethyl-(2-butyl, 2-myristyl)-malonate by fractional distillation under reduced pressure, it was next converted to the free dicarboxylic acid by refluxing with 2 M sodium hydroxide in 95% ethanol at 75–80°C. After the hydrolysis was complete, the reaction was quenched by pouring the free dicarboxylic acid into water that was acidified to pH 2 by the addition of hydrochloric acid and extracting it with chloroform. The chloroform extract was next washed with water and concentrated by rotary evaporation and was sufficiently pure for use without further purification. The free (*dl*)-2-butyl-hexadecanoic acid was formed by decarboxylating the 2-butyl, 2-myristyl-malonic acid by vigorously stirring it in 12 M sulfuric acid at 160°C until the reaction was complete (usually 1–2 h). After allowing the reaction mixture to cool, the mixture was carefully poured onto ice, diluted with water, and extracted with chloroform. The free (*dl*)-2-butyl-hexadecanoic acid was concentrated by rotary evaporation of the chloroform extract and purified by silicic acid chromatography. The (*dl*)-2-alkyl fatty acids were converted to their respective PCs by the acylation of the cadmium chloride complex of L-α-glycerophosphorylcholine using the fatty acid anhydrides and 4-pyrrolidino pyridine as a catalyst as previously described by Patel et al. (1979). However, with those fatty acids that had very large or very highly branched alkyl substituents near the carboxyl group, it was necessary to use substantially larger quantities of the fatty acid (three- to fivefold) and a proportionally larger quantity of catalyst (two- to threefold) and to add a large excess of

N, N-dicyclohexyl carbodiimide, because the reaction proceeded very slowly under the conditions initially described by Patel et al. (1979). The crude PCs were subsequently purified by the silicic acid chromatographic procedures previously used in this laboratory to obtain highly purified samples (Lewis and McElhaney, 1985).

The DSC measurements were performed with a Perkin Elmer (Norwalk, CT) DSC-2C scanning calorimeter equipped with a thermal analysis data station. The samples were prepared by introducing 3–4 mg of the dry lipid into a stainless steel, large volume capsule and adding 50 μl of distilled water. The capsule was subsequently sealed, and hydration of the sample was achieved by repeated heating and cooling of the sample between 95 and −30°C. Once hydrated, DSC heating and cooling thermograms were recorded at 0.31°C/min unless otherwise stated. The data was subsequently analyzed using TADS software and other computer programs developed in this laboratory. After the DSC measurements were complete, the sample capsule was opened and its contents quantified by the lipid phosphorous assay developed by Raheja et al. (1973). ^{31}P-NMR spectra were recorded between 0 and 85°C with a Nicolet (Madison, WI) NT300-WB spectrometer using standard single pulse, direct excitation techniques and the data acquisition and postprocessing parameters previously reported (Lewis et al., 1988).

Small and wide angle x-ray diffraction patterns were recorded with the Princeton (Princeton, NJ) SIV x-ray beam line (Reynolds et al., 1978) or with a similar beam line in which the SIV vidicon area detector was replaced with a slow scan CCD camera. Sample preparation and data methodologies have been described by Lewis et al. (1989). Briefly, dry lipid and deionized water in a typical weight ratio of 1:1 to 1:2 were mechanically mixed in the bottom of a 1.5-mm x-ray capillary, which was then sealed with epoxy cement. The samples were allowed to equilibrate overnight and then were placed in a computer-controlled, thermoelectrically driven temperature stage on the x-ray beamline. The beamline uses point-focus x-ray optics and a quantum-limited area detector with sufficient efficiency that two-dimensional diffraction parameters could be acquired with exposures of 5–10 min. A typical data acquisition protocol consists of stepwise ramping of the temperature of the specimen from −30 to 85°C and back down to −30°C in 5 or 10°C steps every half-hour. Each half-hour period consists of a rapid (≅1 min) temperature step to the desired temperature followed by a 20-min incubation period and the acquisition of a diffraction pattern.

RESULTS

There is now a sizable literature on the effects of variations in acyl chain structure on the thermotropic phase behavior of hydrated PC bilayers (see Menger et al., 1988a, b, and reviews by Silvius, 1982; Lewis and McElhaney, 1992). Despite the fairly extensive structural modifications of the fatty acyl chains described in such studies, the PC molecules studied to date all form lamellar phases when dispersed in excess water up to at least 100°C. With most of the PC molecules studied so far, the structure of the acyl chains have been modified at either their hydrophobic termini (see Silvius, 1982; Lewis and McElhaney, 1992 and references cited therein) or in the middle region of the hydrocarbon chain (e.g., Barton and Gunstone, 1975), and there have been very few studies of the properties of PC bilayers with acyl chain modifications near the ester carbonyl moiety (for examples, see Barton and Gunstone, 1975; Brezesinski et al., 1983, 1987; Nuhn et al., 1986). Given this, we have synthesized a number of (*dl*)-2-substituted fatty acids and their corresponding 1,2-diacyl PCs and have characterized their thermotropic phase behavior by DSC, x-ray diffraction, and ^{31}P-NMR spectroscopy. In particular, the tendency of these PC molecules to form nonlamellar phases has been examined from the perspectives of both the length and structure of the 2-alkyl substituent and the length of the main acyl chain. A

TABLE 1 Chemical structures of the hydrophobic groups substituted at the 2-position of the fatty acyl chains

Substituent	Chemical Structure
Methyl	—CH_3
Ethyl	—CH_2—CH_3
Propyl	—CH_2—CH_2—CH_3
2-Methyl Propyl (iso butyl)	—CH_2—$CH(CH_3)$—CH_3
Butyl	—CH_2—CH_2—CH_2—CH_3
2-Methyl Butyl (sec pentyl)	—CH_2—$CH(CH_3)$—CH_2—CH_3
3-Methyl Butyl (iso pentyl)	—CH_2—CH_2—$CH(CH_3)$—CH_3
3,3-Dimethyl Butyl	—CH_2—CH_2—$C(CH_3)_2$—CH_3
2-Ethyl Butyl	—CH_2—$CH(C_2H_5)$—CH_2—CH_3
Pentyl	—CH_2—CH_2—CH_2—CH_2—CH_3
Cyclohexyl Methyl	—CH_2—C_6H_{11}
Benzyl	—CH_2—C_6H_5

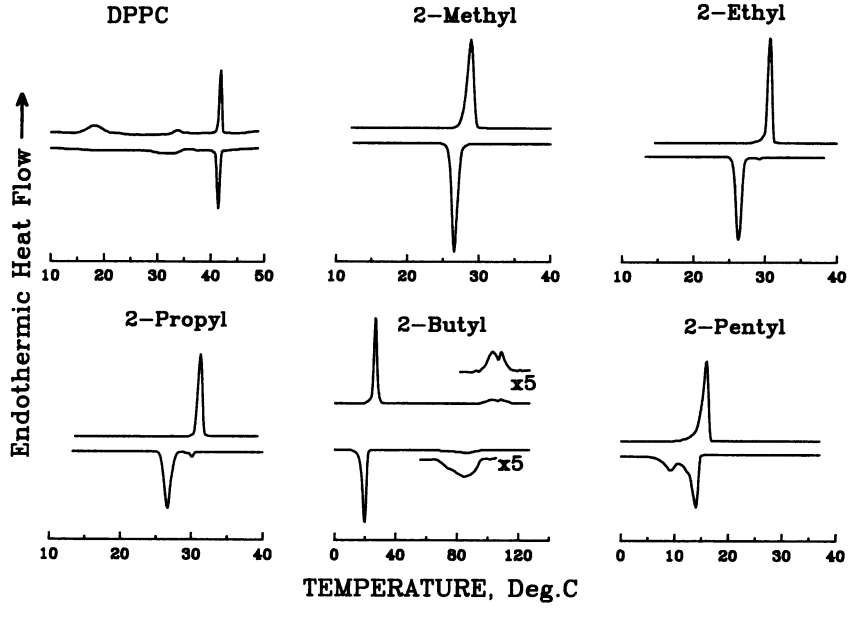

FIGURE 1 DSC thermograms of DPPC, di-(*dl*)-2-methyl-hexadecanoyl PC, di-(*dl*)-2-ethyl-hexadecanoyl PC, (*dl*)-2-propyl-hexadecanoyl PC, di-(*dl*)-2-butyl-hexadecanoyl PC, and di-(*dl*)-2-pentyl-hexadecanoyl PC. Unless otherwise stated, the DSC themograms were all acquired at a scan rate of 18.75°C/h.

list of the chemical structures of the 2-alkyl substituent groups used in this study is presented in Table 1.

Effects of substituent length

Figs. 1 and 2 show DSC thermograms and ^{31}P-NMR spectra illustrating the thermotropic phase behavior of aqueous dispersions of DPPC, di-(*dl*)-2-methyl-hexadecanoyl PC, di-(*dl*)-2-ethyl-hexadecanoyl PC, di-(*dl*)-2-propyl-hexadecanoyl PC, di-(*dl*)-2-butyl-hexadecanoyl PC, and di-(*dl*)-2-pentyl-hexadecanoyl PC. As expected from previous studies (see Lewis et al., 1987 and references cited therein), DPPC dispersions that have been annealed at low temperatures for several days exhibit three heating endothermic transitions, which correspond to the subtransition, pretransition, and main or gel/liquid-crystalline phase transition in order of increasing temperature, respectively. Upon cooling, the main phase transition is found to be freely reversible with little or no hysteresis, the pretransition is observable but exhibits a modest cooling hysteresis, and the subtransition is not observed, because it is a considerably slower process. Also, as shown in Fig. 2, DPPC exhibits ^{31}P-NMR spectra that are compatible with the existence of lamellar structures over the entire temperature range examined (0–100°C). In contrast, the di-(*dl*)-2-alkyl-hexadecanoyl PCs all exhibit single heating endotherms that occur at a lower temperature than does the main phase transition of DPPC (see Table 2 *A*). With the di-(*dl*)-2-alkyl-hexadecanoyl PCs, the gel/liquid-crystalline phase transition is also reversible, but, unlike DPPC, these transitions exhibit some cooling hysteresis (range 2–7°C). For those lipids with the shorter chain substituents (i.e., methyl, ethyl, and propyl), the ^{31}P-NMR spectra are powder patterns arising from predominantly lamellar assemblies of phospholipid molecules undergoing axially symmetric motion both above and below the main phase transition temperature (see Seelig, 1978). However, at temperatures above the gel/liquid-crystalline phase transition, their ^{31}P-NMR spectra each contain a small "isotropic peak" of spectral intensity near 2 ppm downfield. This peak is not present in the gel-phase spectra of these lipids and increases in intensity with increases in temperature. Sharp isotropic peaks at this frequency are observed when the three components of the ^{31}P shift tensor have been averaged on the ^{31}P-NMR time scale. In phospholipid dispersions, such spectra are usually observed when: 1) small unilamellar vesicles that can undergo fact tumbling on the ^{31}P-NMR time scale are present, 2) there has been hydrolytic degradation leading to the formation of micellar assemblies, or 3) structurally complex three-dimensional lipid assemblies, such as cubic phases, are formed. The presence of a population of small unilamellar vesicles seems improbable in the light of the fact that the isotropic peak disappears from the ^{31}P-NMR spectrum when the samples are cooled to "gel-phase" temperatures, and we have determined that hydrolytic degradation did not occur during these experiments. Thus these data suggest that small amounts of nonlamellar phases are formed when aqueous dispersions of these di-(*dl*)-2-alkyl-hexadecanoyl PCs are heated above their main phase transition temperatures. However, because the spectroscopic data indicate that these di-(*dl*)-2-alkyl-hexadecanoyl PCs are predominantly lamellar at all temperatures examined, their tendencies to form nonlamellar phases must be relatively small under our experimental conditions. Nevertheless, these di-(*dl*)-2-alkyl-hexadecanoyl PCs are clearly closer to their limits of lamellar phase stability than are their unsubstituted analogues.

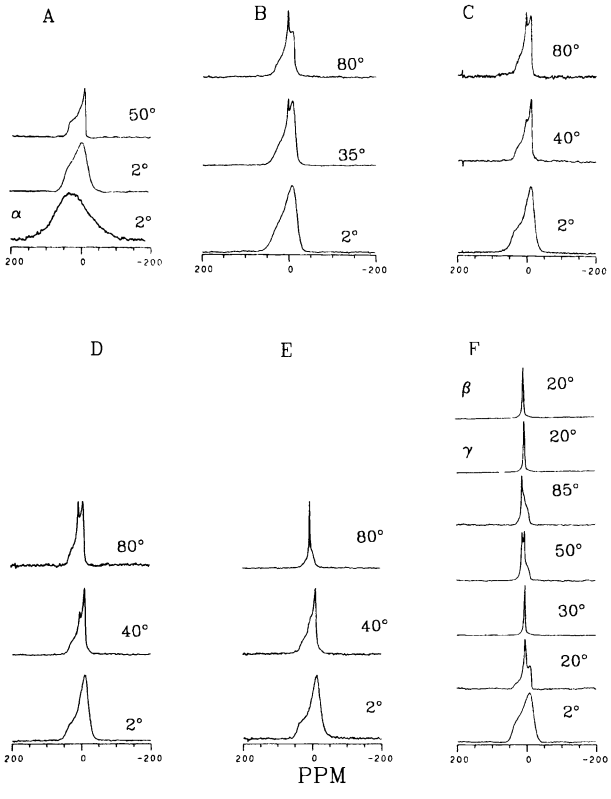

FIGURE 2 Proton decoupled ^{31}P-NMR spectra of (*A*) DPPC, (*B*) di-(dl)-2-methyl-hexadecanoyl PC, (*C*) di-(*dl*)-2-ethyl-hexadecanoyl PC, (*D*) di-(*dl*)-2-propyl-hexadecanoyl PC, (*E*) di-(*dl*)-2-butyl-hexadecanoyl PC, and (*F*) di-(*dl*)-2-pentyl-hexadecanoyl PC. The spectra were obtained at the temperatures indicated and, unless otherwise stated, they were acquired in a sequence corresponding to the heating mode of the DSC experiment. α DPPC sample incubated at 0–4°C for 7 days; β sample cooled from 85°C; and γ sample cooled from 30°C.

It is also evident that the predisposition to form nonlamellar phases increases dramatically when the length of the 2-alkyl substituent is increased beyond 3 carbon units (see Fig. 2). With di-(*dl*)-2-butyl-hexadecanoyl PC, the DSC thermograms (Fig. 1) show a main phase transition at temperatures near 27°C and additional thermal events at elevated temperatures (between 80 and 110°C). Also, at higher temperatures the isotropic peak becomes the dominant feature of its ^{31}P-NMR spectrum, suggesting that considerable amounts of nonlamellar structures are formed. The cooling thermograms exhibited by di-(*dl*)-2-butyl-hexadecanoyl PC (see Fig. 1) indicate that these thermotropic events are reversible, although they exhibit significant cooling hysteresis. In the

TABLE 2 Thermodynamic characterization of the di-(*dl*)-2-alkyl substituted phosphatidylcholines

(*dl*) 2-Alkyl Group	*T_m (°C) Heating	*T_m (°C) Cooling	‡ ΔH_{cal} (Kcal/mol)	§ Comments
A) di-(*dl*)-2-Alkyl-hexadecanoyl PCs				
None (DPPC)	41.4	41.3	7.7	Lamellar at all temperatures measured (0–85°C)
Methyl	28.5	26.5	8.6	Predominantly lamellar with small populations of cubic structures at $T > T_m$
Ethyl	30.8	26.4 (29.2)	8.7	Predominantly lamellar with small populations of cubic structures at $T > T_m$
Propyl	31.4	36.7 (30.2)	8.9	Predominantly lamellar with small populations of cubic structures at $T > T_m$
Butyl	26.6	19.4	8.3	Predominantly lamellar at T > 65°C Predominantly cubic at T > 85°C
Pentyl	16.0	13.2 (9.4)	7.6	Predominantly lamellar at T < 25°C Cubic at 30° < T < 45°, H_{II} at T > 60°C
2-methyl,propyl	26.3	24.9 (22.9)	7.9	Lamellar at T<60°C Cubic at T ≅ 85°C
2-methyl,butyl	17.5	12.1	7.1	Lamellar at T < 25°, Cubic (Ia3d) at 25° > T > 50° Cubic (Pn3m) at T > 70°
3-methyl,butyl	18.5	16.5 (12.5)	7.1	Predominantly lamellar at T_m < T < 30° Cubic at T > 40, H_{II} when rapidly heated to 85°
3,3-dimethyl-butyl	14.6	11.4	7.9	Lamellar only at $T < T_m$ Cubic at T = 15°C, H_{II} at T > 20°C
cyclohexyl,methyl	6.4	4.2	7.9	Lamellar only at $T < T_m$ H_{II} at $T > T_m$
2-ethyl,butyl	9.8	9.2 (16.8)	5.4	Predominantly lamellar at T_m < T < 20° Cubic at 20° < T < 30°, H_{II} at T > 40°C
2-benzyl	18.5	16.8 (15.8)	7.7	Lamellar at all temperatures measured (0–85°C)
B) di-(*dl*)-2-Alkyl-Dodecanoyl PCs				
None (DLPC)	−2.1	−3.1 (−2.1)	1.8	Lamellar at all temperatures measured (0–85°C)
Benzyl	<−50	<−50	¶ nd	Lamellar at T < 65°C, predominantly cubic at T > 80°C
C) di-(*dl*)-2-Alkyl-Tetradecanoyl PCs				
None (DMPC)	23.9	23.8	5.9	Lamellar at all temperatures measured (0–85°C)
Butyl	0.9	−2.2 (0.75)	5.8	Predominantly lamellar at T < 70°C Cubic at T > 80°C
Cyclohexyl,methyl	−36.1	−40.3	nd	H_{II} at all temperatures measured (0–85°C)
2-benzyl	−9.4	−10.1	5.6	Predominantly lamellar at T < 60°C with small populations of cubic structures at higher temperatures
D) di-(*dl*)-2-Alkyl-Octadecanoyl PCs				
None (DSPC)	55.3	55.2	9.8	Lamellar at all temperatures measured (0–85°C)
Butyl	42.4	41.4	10.5	Lamellar at all temperatures measured (0–85°C)
Cyclohexyl,methyl	24.5	21.5 (20.8)	10.5	Lamellar only at $T < T_m$, H_{II} at $T > T_m$

* T_ms of minor transitions listed in brackets.
‡ Refers only to the chain-melting phase transition.
§Temperatures from ^{31}P-NMR experiments heating from $T < T_m$.
¶ Not determined.

case of di-(*dl*)-2-pentyl-hexadecanoyl PC, DSC reveals a single heating endothermic transition at temperatures near 16°C with no calorimetric evidence for further thermal changes. However, the ^{31}P-NMR spectroscopic data indicate that structural changes are occurring at temperatures above the chain-melting transition temperature. At low temperatures the sample exhibits a powder pattern that is compatible with the existence of a L_β-type gel-phase bilayer, but at all temperatures above the main transition temperature there is evidence for a significant nonlamellar component in the spectrum. At 20°C there seem to be comparable proportions of liquid-crystalline bilayer and isotropic components in the ^{31}P-NMR spectrum, but the bilayer component disappears completely at 30°C. For this particular di-(*dl*)-2-alkyl-hexadecanoyl PC, our x-ray diffraction studies indicate that the isotropic structure formed is consistent with cubic phases of the Pn3m or Pn3 space groups (see below). Interestingly, the cubic phase formed does not reconvert to the lamellar liquid-crystalline phase when recooled to temperatures near 15°C but seems to convert directly to the gel phase when cooled to lower temperatures. This type of behavior is remarkably similar to the metastable behavior observed with the Pn3m cubic phase formed when dioleoyl phosphatidylethanolamine dispersions have been rapidly recycled (≅200 ×) through their L_α/H_{ii} transition temperature range (Shyamsunder et al., 1988).

The ^{31}P-NMR spectroscopic data also show that further structural changes occur when samples of di-(*dl*)-2-pentyl-hexadecanoyl PC are heated to temperatures near 85°C, despite the fact that there is no calorimetric evidence for structural changes over this temperature range. At temperatures near 50°C the spectrum broadens and assumes the appearance of a two-component system composed of an isotropic component and a broader powder pattern, which is of re-

duced anisotropy and reversed asymmetry when compared with that of the lamellar liquid-crystalline phase. With further heating the isotropic component eventually disappears completely, leaving a powder pattern that is generally indicative of the formation of the inverted hexagonal phase. Interestingly, the structural changes that occur between 30 and 85°C are fully reversible. That these phase transitions were not detected by DSC suggests that the structural changes concerned are weakly energetic and/or poorly cooperative thermotropic events.

Effects of substituent bulk and rigidity

Having established that the predisposition of lipids with normally "bilayer-preferring polar headgroups" to form nonbilayer phases can be dramatically increased by the introduction of sufficiently long substituents near the ester carbonyl groups of the fatty acyl chain, we next examined the effects of changes in the bulk and rigidity of the substituent group. Illustrated in Figs. 3 and 4 are DSC thermograms and ^{31}P-NMR spectra of some di-(*dl*)-2-alkyl-hexadecanoyl PCs in which the nominal length of the substituent group is 4 carbon atoms. With these particular lipids, the substituent group is made bulkier by subsidiary branching. A comparison of the thermotropic properties of these samples with those of di-(*dl*)-2-butyl-hexadecanoyl PC (see Figs. 1 and 2), which has an unbranched substituent group of similar length, reveals that subsidiary branching of the substituent group results in further reductions in the gel/liquid-crystalline phase transition temperatures (see data compiled in Table 2). This is most likely the result of further destabilization of the gel phase caused by the presence of the bulkier substituent groups. From the ^{31}P-NMR spectra it is also clear that both the absolute temperature and the reduced temperature (relative to the main phase transition temperature) at which these lipids form nonlamellar phases (whether isotropic or H_{II}) are considerably lower than those of di-(*dl*)-2-butyl-hexadecanoyl PC. Moreover, the type of nonlamellar phase formed and the predisposition of the lipid molecule to form such phases are also affected by the position of the subsidiary group on the 2-alkyl substituent. Thus for the lipid with 3-methyl-butyl (iso-pentyl) substituted fatty acyl chains, DSC heating thermograms acquired at fast scan rates reveal an additional thermal event near 57°C, which, on the basis of the ^{31}P-NMR spectroscopic data, is consistent with a cubic/H_{II} phase transition. As shown in Fig. 4 *B*, the ^{31}P-NMR spectra indicate that the conversion of the lamellar phase to an isotropic phase is complete at temperatures near 40°C, whereas the spectrum acquired at 85°C shows that there is coexistence of both isotropic and H_{II} phases. Interestingly, however, our ^{31}P-NMR spectroscopic studies also suggest that there is a kinetic component to this process, inasmuch as the partial conversion to the H_{II} phase of this particular lipid is only observed if the sample is heated very quickly. Thus we suspect that complete conversion to the H_{II} phase may occur in the fast scanning DSC experiment but not in the NMR spectroscopic experiment because of the greater thermal inertia of the NMR sample. In the case of the di-(*dl*)-2-(2-methyl-butyl)-hexadecanoyl PC (sec-pentyl substituted PC), however, it is evident that this lipid is less prone to form a nonlamellar phase than is the iso-pentyl substituted analogue, because the conversion of the lamellar phase to the cubic phase occurs at somewhat higher temperatures (whether viewed on the absolute or reduced temperature scales) and no H_{II} phase is formed.

Our studies of the sec-pentyl-substituted PC also suggest that this lipid may form two distinct, thermotropically induced cubic phases. A close inspection of the data shown in Fig. 4 *A* shows that the isotropic ^{31}P-NMR signal obtained at 40°C is broader than that obtained at 85°C. Moreover, this is not simply the result of a temperature-dependent line narrowing caused by the increased mobility of the lipid mol-

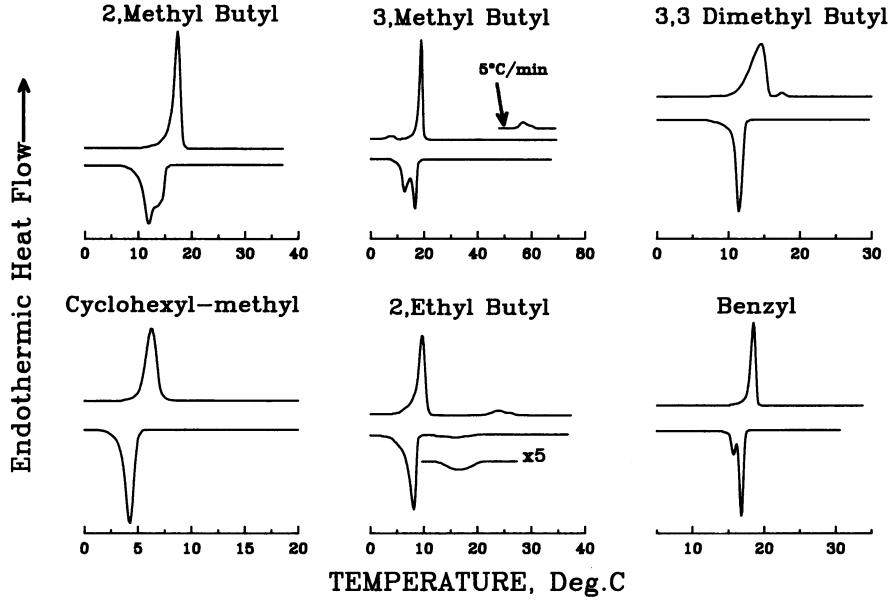

FIGURE 3 DSC thermograms of di-(*dl*)-2-(2-methyl butyl)-hexadecanoyl PC, di-(*dl*)-2-(3-methyl butyl)-hexadecanoyl PC, di-(*dl*)-2-(-3,3-dimethyl butyl)-hexadecanoyl PC, di-(*dl*)-2-(cyclohexyl methyl)-hexadecanoyl PC, di-(*dl*)-2-(2-ethyl butyl)-hexadecanoyl PC, and di-(*dl*)-2-benzyl-hexadecanoyl PC. Unless otherwise stated, the DSC themograms were all acquired at a scan rate of 18.75°C/h.

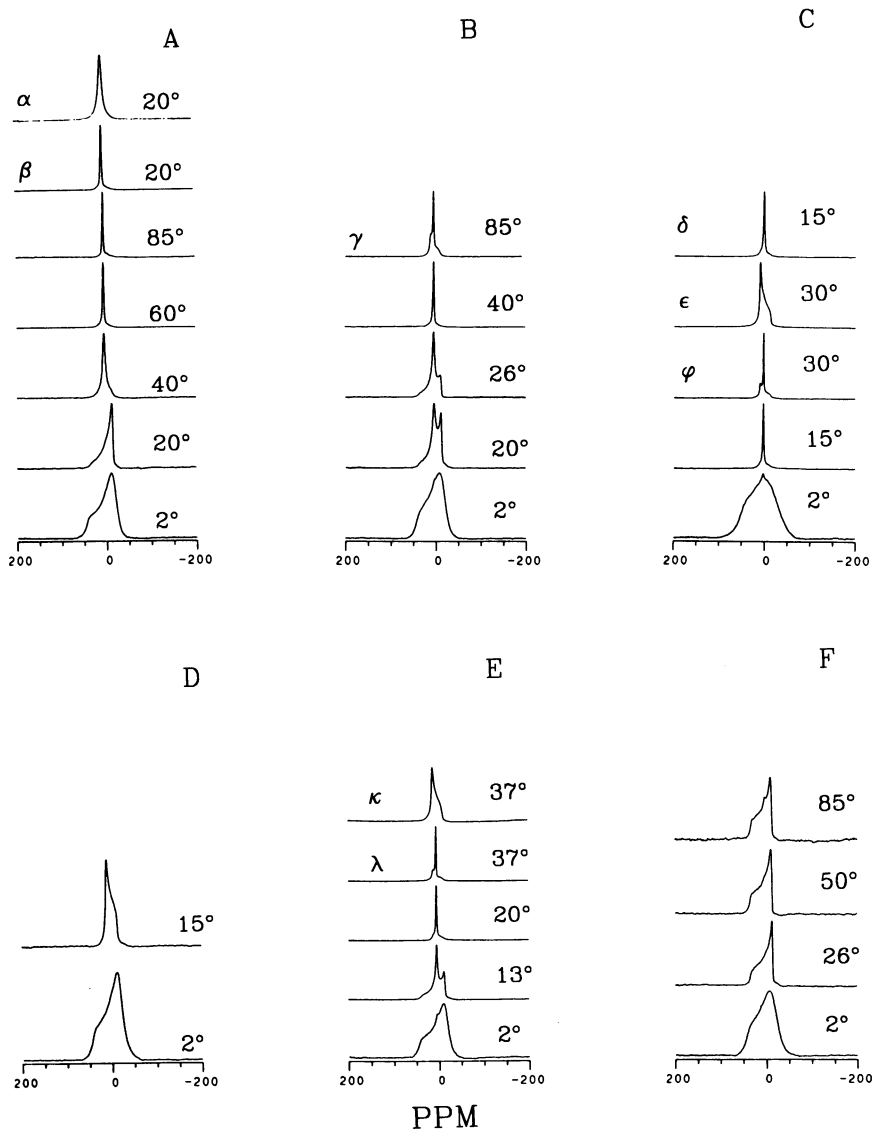

FIGURE 4 Proton decoupled ^{31}P-NMR spectra of (*A*) di-(*dl*)-2-(2-methyl butyl)-hexadecanoyl PC, (*B*) di-(*dl*)-2-(3-methyl butyl)-hexadecanoyl PC, (*C*) di-(*dl*)-2-(-3, 3-dimethyl butyl)-hexadecanoyl PC, (*D*) di-(*dl*)-2-(cyclohexyl methyl)-hexadecanoyl PC, (*E*) di-(*dl*)-2-(2-ethyl butyl)-hexadecanoyl PC, and (*F*) di-(*dl*)-2-benzyl-hexadecanoyl PC. The spectra were obtained at the temperatures indicated and, unless otherwise stated, were acquired in a sequence corresponding to the heating mode of the DSC experiment. α sample cooled from 40°C; β sample cooled from 85°C; γ sample quickly heated from 2°C; δ sample cooled from 15°C; ϵ sample quickly heated from 2°C; φ sample heated from 15°C; κ sample quickly heated from 2°C; and λ sample PC heated from 15°C.

ecules at higher temperatures. As is also shown in Fig. 4 *A*, the line width of the isotropic NMR signal obtained at a common data acquisition temperature of 20°C differs according to the thermal history of the sample. For samples that were preequilibrated at 2°C, heated to 40°C, and recooled to 20°C, the width of the isotropic signal is significantly broader than that obtained from a sample that was cooled to 20°C from a temperatures near 85°C. This difference in the line widths is a reflection of structural differences as confirmed by a visual inspection of the sample used for NMR spectroscopy (see Fig. 5). When warmed from the gel phase to temperatures near 20°C, the sample appears as an opaque dispersion typical of most lamellar lipid dispersions. However, when warmed to 40°C the appearance of the sample changes to that of a transparent/translucent viscous liquid, which is less dense than the bulk aqueous phase. This change in the appearance of the sample is not the result of dehydration, because our gravimetric analyses of the "transparent viscous liquid" that floated out of the bulk aqueous phase indicate that it consists of 70–85% water. Upon heating to temperatures near the boiling point of water, the appearance of the sample changed further and assumed that of a white translucent "solid-like" material, which was firm to the touch. Our gravimetric analyses of the latter showed that it also consists of 70–85% water.[1]

[1] Note that these gravimetric estimates of water content probably include bulk water trapped in defects or between local domains of the aggregate concerned. Therefore these values should be considered as the upper limit of the actual water content of these phases.

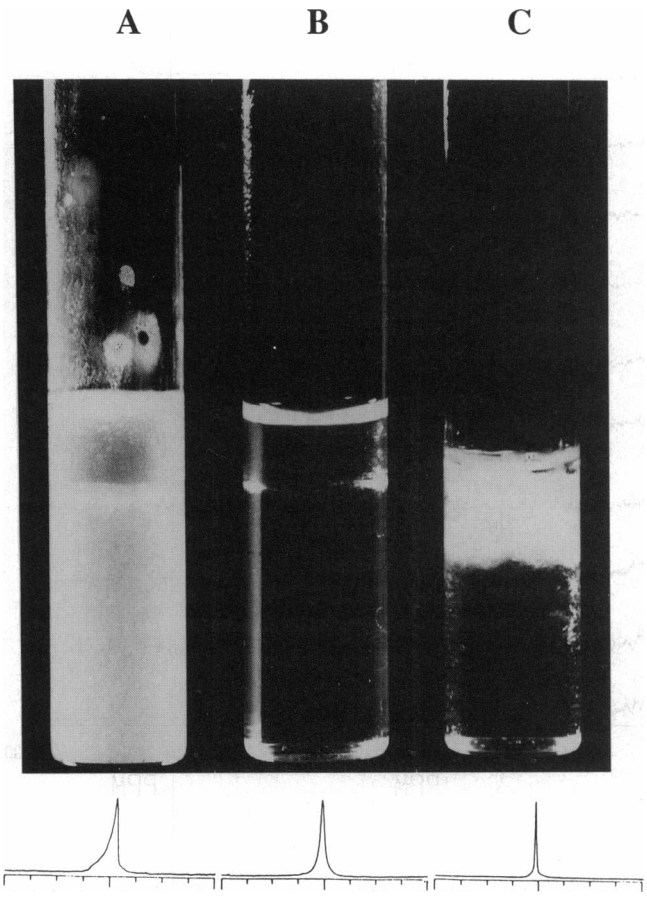

FIGURE 5 Photographs and ^{31}P-NMR spectra of aqueous dispersions of di-(*dl*)-2-(sec-pentyl)-hexadecanoyl PC. The photographs were all taken at 20°C. (*A*) Sample warmed to 20°C from 2°C (Lamellar L_α phase); (*B*) Sample recooled to 20°C after warming sample A to 40°C (cubic phase Ia3d); and (*C*) Sample recooled to 20°C after heating to 85°C (cubic phase Pn3m).

The changes in the appearance and the consistency of the sample are clearly the result of significant structural changes. The nature of the structures formed was such that the appearance of the transparent viscous liquid is correlated with the broad isotropic signal, whereas the narrower signal is correlated with the appearance of the hard translucent solid. The existence of two isotropic ^{31}P-NMR signals suggests that one or more structurally distinct types of cubic phases may have been transiently formed by some of the other cubic phase-forming PCs examined (see x-ray diffraction data below). This phenomenon may explain the kinetic aspects of the behavior of di-(*dl*)-2(iso-pentyl) hexadecanoyl PC noted above.

We also find that additional branching of the substituent group results in a further destabilization of the gel phase and an increased tendency to form nonlamellar phases. For example, with (*dl*)-2-(3, 3,dimethyl-butyl)-hexadecanoyl PC, the presence of the added methyl group at C3 of the butyl chain results in a further lowering of the chain-melting transition temperature by some 4°C (see Table 2 *A* and compare with (*dl*)-2-(iso-pentyl)-hexadecanoyl PC) and a dramatic lowering of the lamellar/nonlamellar phase transition temperature. In fact this particular lipid is the only one of the (*dl*)-2-(alkyl)-hexadecanoyl PCs synthesized that exhibits ^{31}P-NMR spectra consistent with the formation of an ordered L_c-like phase at low temperatures. As is evident from Fig. 4 *C*, the heating endothermic transition centered near 14°C seems to coincide with a direct conversion from a subgel (L_c-like) phase to a nonlamellar phase. Apparently, the L_β and L_α lamellar phases of this particular lipid are not thermodynamically favored at any temperature. It is also apparent from the data in Fig. 4 that there is a kinetic component to the behavior of this lipid, the form of which is similar to but not identical with that observed with (*dl*)-2-(iso-pentyl)-hexadecanoyl PC. To further investigate the phase transition kinetics, we designed a time-resolved NMR experiment in which a sample of the lipid was equilibrated at 0°C and quickly introduced into the NMR probe, which was preequilibrated at 15°C. Spectra were acquired every 2 min (coaddition of 32 transients) as the sample warmed to 15°C, and the results are shown in Fig. 6. Initially, the broad powder pattern characteristic of the L_c phase was observed (Fig. 6, spectra 1–4), but this soon collapsed to form the axially symmetric pattern expected of the lamellar L_α phase (see Fig. 6, spectra 6 and 7). However, a population of nonlamellar phase material was also present, as evidenced by the isotropic peak near 2 ppm downfield (see Fig. 6, spectra 6 and 7). With time, these events are superseded by formation of the inverted hexagonal phase, which is virtually complete some 20 min after the start of the experiment (see Fig. 6, spectra 10 and 11). The fact that the H_{II} phase is formed at 15°C seems surprising in view of the fact that the data shown in Fig. 4 *C* clearly indicate that a cubic phase (assigned as cubic Pn3m/Pn3 by x-ray diffraction, see below) is the thermodynamically favored phase at this temperature. However, with prolonged incubation at 15°C, there is a slow build-up of an isotropic component in the spectrum (see Fig. 6, compare spectra 10 and 30), which is consistent with the slow conversion of the H_{II} phase to a cubic phase. Indeed, as is evident from Fig. 5 *C*, complete conversion to the cubic phase occurs when the sample is fully equilibrated under these conditions. It seems highly improbable that the isotropic signal transiently observed when the L_c phase decomposed arises from the same cubic phase, which is thermodynamically favored at 15°C. Thus it is possible that the "isotropic structure" (possibly a cubic phase) that is transiently formed between the lamellar and H_{II} phases of this lipid may be structurally distinct from the Pn3m (or Pn3 phase), which is stable at 15°C.

To investigate the effects of the rigidity of the substituent group, we examined the thermotropic phase properties of di-(*dl*)-2-(cyclohexyl-methyl)-hexadecanoyl PC, its open chain analogue di-(*dl*)-2-(2-ethyl-butyl)-hexadecanoyl PC, and the aromatic ring analogue di-(*dl*)-2-benzyl-hexadecanoyl PC (see Figs. 3 and 4 and data compiled in Table 2 *A*). In the case of the cyclohexyl-methyl substituted lipid, a single heating endothermic transition is observed by DSC, and this was identified as a lamellar gel/H_{II} phase transition by both ^{31}P-NMR spectroscopy and x-ray diffraction. Evidently, the presence of a large rigid group at C2 of the fatty

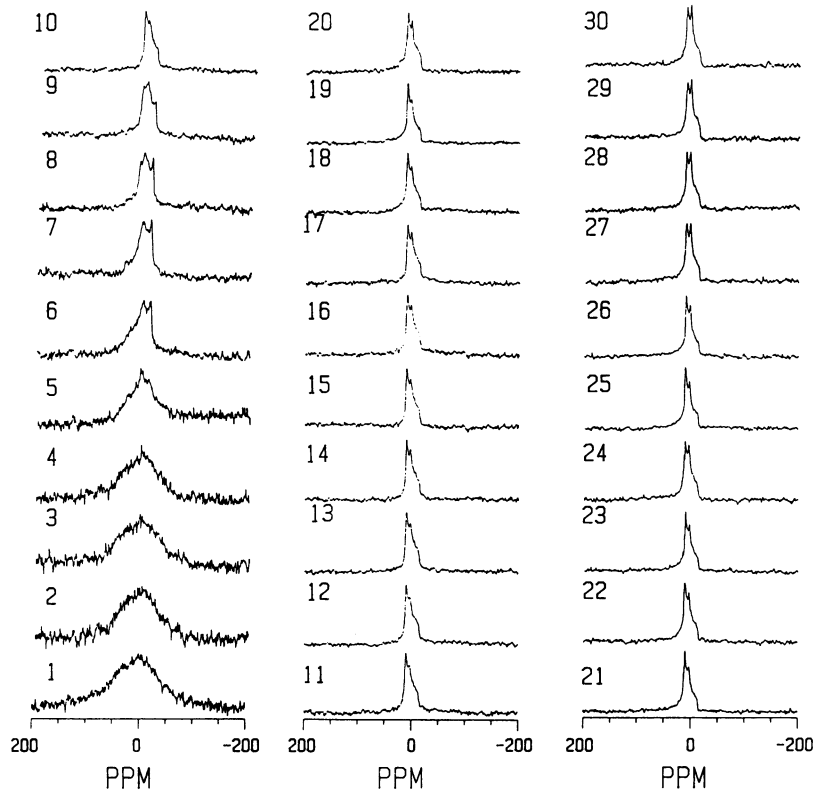

FIGURE 6 Proton decoupled ^{31}P-NMR spectra of a sample of di-(*dl*)-2-(3, 3-dimethyl-butyl)-hexadecanoyl PC acquired as a function of time as the sample warmed from 0°C to 15°C.

acyl chain is a potent promoter of nonlamellar phase formation. However, in the case of the aromatic ring analogue (di-(*dl*)-2-benzyl-hexadecanoyl PC), we found no evidence for the formation of any form of nonlamellar phase. From the considerable lowering of the gel/liquid-crystalline phase transition temperature (see DSC data in Fig. 3 and Table 2*A*), it is clear that the presence of the benzyl groups does disrupt the lateral interactions of the hydrocarbon chains, but evidently its effects are not severe enough to destabilize this particular bilayer with respect to a nonlamellar phase. We do find, however, that the 2-benzyl substituent can promote the formation of an inverted nonlamellar structure if the length of the main acyl chain is shortened (see Fig. 7 and Table 1, *B* and *C*). Evidently, the size and rigidity of the benzyl group are not the only factors that need to be considered. However, that the rigidity of the substituent group is a significant factor is clearly evidenced by a comparison of the behavior of the cyclohexylmethyl-substituted lipid with that of the 2-ethyl-butyl-substituted analog. With the latter the branched substituent group is of comparable size to the cyclohexyl-methyl group, but it is considerably more flexible, because the carbon atoms are not constrained to form an alicyclic ring. A comparison of the thermodynamic and spectroscopic data suggests that the more flexible 2-ethyl-butyl group is less disruptive of gel-state packing (note its higher chain-melting phase transition temperature, see Fig. 4 *E* and Table 2) and is also a less potent promoter of nonlamellar phase formation. Unlike the cyclohexylmethyl analogue, there is a small temperature range (10–20°C) in which the lamellar L_α and cubic phases of this lipid coexist and the H_{II} phase is only stable at temperatures above 25°C (see Fig. 4 *E*). It is also interesting to note that there is a kinetic component to the behavior of this lipid similar to that observed with di-(*dl*)-2-(3, 3,dimethyl-butyl)-hexadecanoyl PC (see Fig. 4 *E*).

Effect of the length of the main acyl chain

To evaluate how the effect of the (*dl*)-2-alkyl substitutions are moderated by the length of the main acyl chain, we also examined the thermotropic phase behavior of the di-(*dl*)-2-butyl, di-(*dl*)-2-(cyclohexylmethyl), and di-(*dl*)-2-benzyl alkanoyl PCs with comparable variation in their main chain lengths. These three substituent groups span the wide range of "non-lamella-inducing" potencies observed in our studies of the di-(*dl*)-2-alkyl-hexadecanoyl PCs. With each series of lipids, the thermodynamic data (see Tables 1 and 2) show the expected increase in the gel/liquid-crystalline phase transition temperature with increases in the main acyl chain length. Moreover, the "disruptive" effects of the 2-alkyl substituent groups decrease markedly with increases in the length of the main acyl chain, as indicated by the progressively smaller decrease in T_m observed (see Table 3). Our ^{31}P-NMR spectroscopic studies show that the stability of these PC bilayers with respect to an inverted nonlamellar phase is chain-length dependent (see Fig. 7). As shown in Table 2 and Fig. 7, all of the cyclohexylmethyl-substituted homologues form H_{II} phases, whereas some of the homologues of the 2-butyl and the 2-benzyl-substituted PCs form inverted cubic phases. However, the remarkable observation is that the propensity of these PCs to form inverted nonlamellar phases seems to

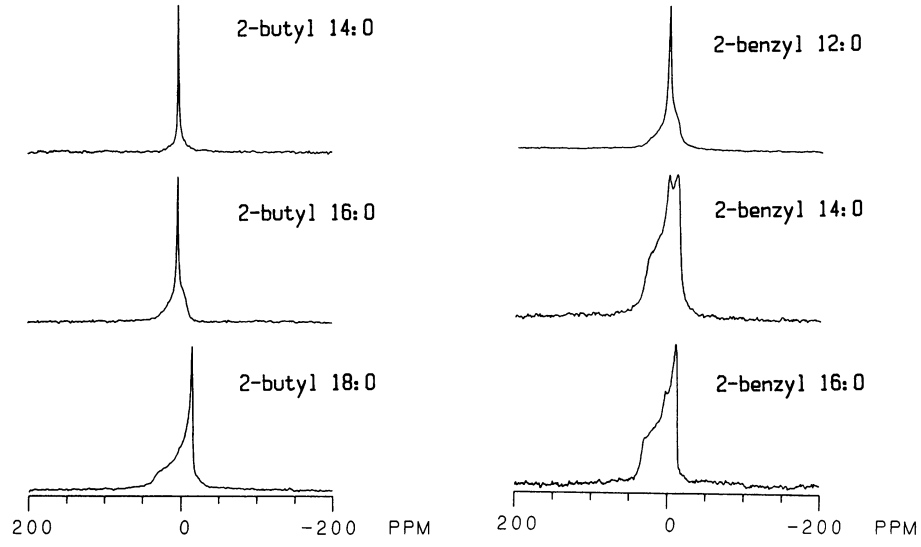

FIGURE 7 Proton-decoupled ^{31}P-NMR spectra of di-(*dl*)-2-butyl- (*left panel*) and di-(*dl*)-2-benzyl-substituted analogues of the homologous series of normal saturated diacyl PCs. The spectra shown were all acquired at 85°C.

decrease with increases in acyl chain length. This is vividly illustrated by our studies of the di-(*dl*)-2-benzyl and di-(*dl*)-2-butyl alkanoyl PCs, which clearly show that at a constant temperature of 85°C, the extent of conversion to the cubic phase decreases as the length of the main fatty acyl chain increases (see Fig. 7). This is in marked contrast to the diacyl and dialkyl PEs (Seddon et al., 1983, 1984; Lewis et al., 1989, Lewis and McElhaney, 1993) and the monoglucosyldiacylglycerols (Mannock et al., 1988, 1990; Mannock and McElhaney, 1991) for which increases in acyl chain length result in a decrease of the lamellar to nonlamellar phase transition temperature. The data presented in Fig. 7 and Table 3 also show that the benzyl substituent does have some capacity to induce the formation of a nonlamellar phase, though it is considerably less effective at so doing than is the cyclohexyl methyl group or, indeed, many of the other hydrophobic groups tested.

X-ray diffraction studies

In this study ^{31}P-NMR spectroscopy was used to characterize the thermally induced structural changes that occur with these novel lipids and for a preliminary assignment of the structure of the phases formed. However, it is well known that structural assignments on the basis of unsupported ^{31}P-NMR spectroscopic data can only be considered tentative. Indeed, it has even been shown that it is theoretically feasible for lamellar phase phospholipids to exhibit spectra that are generally considered to be typical of cubic and H_{II} phases, provided that appropriate restrictions are placed on the motion of the phosphate group (Thayer and Kohler, 1981). To our knowledge this has not been observed experimentally, and indeed we know of no instance in which tentative phase assignments on the basis of ^{31}P-NMR spectroscopy have been shown to be incorrect (see Tilcock et al., 1986). However, because in these studies we have made major modifications to a critical region of the lipid bilayer, one cannot be certain that the above generalizations will hold true with these novel lipids. Thus, the x-ray diffraction patterns of some of these lipids were examined to confirm the tentative phase assignments made by ^{31}P-NMR spectroscopy and to explore certain aspects of the thermotropic phase behavior of some of the novel compounds used in this study.

TABLE 3 Effect of main acyl chain length on the depression in T_m caused by (*dl*)-2-alkyl substituent groups

* Acyl Chain Length	Depression in T_m (°C)		
	Butyl	Cyclohexyl,methyl	Benzyl
12	‡ nd	‡nd	>50
14	23.0	60.0	33.3
16	14.8	35.0	22.9
18	12.9	30.8	‡nd

* Number of carbons forming the main acyl chain.
‡ Not determined.

The spacings corresponding to the lowest order diffraction peaks (i.e., the lamellar repeat distances for lamellar phases) were determined from small angle x-ray diffraction studies of selected di-(*dl*)-2-alkyl-alkanoyl PCs and are plotted as a function of temperature in Fig. 8. The data suggest that these lipids form hydrated bilayers that differ significantly from those formed by normal PC bilayers. First, the plots show a major discontinuity near 0°C upon heating and near −10°C upon cooling (see Fig. 8). These discontinuities probably arise from swelling (shrinkage) of the interlamellar water coincident with the melting (freezing) of the bulk aqueous phase and are not the result of lipid phase changes. Changes of this magnitude are atypical of normal glycerolipid bilayers, for which considerably smaller changes in the dimensions of the interlamellar water layers are usually observed when the bulk aqueous phase freezes or melts. Second, for the alkyl-substituted lipids that form a liquid-crystalline phase at temperatures just above T_m, the long spacings observed at temperatures just below and just above their respective L_β/L_α phase transitions (See Table 4) are smaller than those of the unsubstituted analogue DPPC, for which the

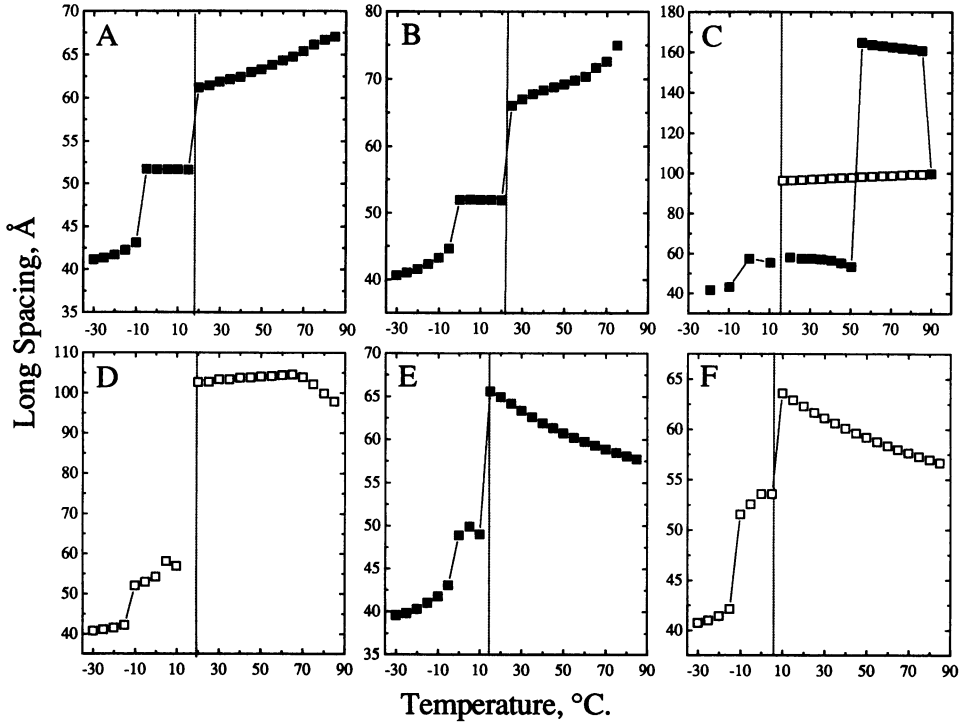

FIGURE 8 Temperature dependence of the long spacings of aqueous dispersions of selected di-(*dl*)-2-alkyl-hexadecanoyl PCs. (*A*) di-(*dl*)-2-Benzyl-hexadecanoyl PC (heating); (*B*) di-(*dl*)-2-Butyl-hexadecanoyl PC (heating); (*C*) di-(*dl*)-2-(2-methyl butyl)-hexadecanoyl PC (heating and cooling); (*D*) di-(*dl*)-2-(3-methyl butyl)-hexadecanoyl PC (cooling); (*E*) di-(*dl*)-2-(3, 3-dimethyl butyl)-hexadecanoyl PC (heating); and (*F*) di-(*dl*)-2-(cyclohexyl methyl)-hexadecanoyl PC (cooling). Open symbols, cooling experiments; filled symbols, heating experiments; and the dashed lines indicate the hydrocarbon chain-melting phase transition temperatures as determined from DSC heating-mode experiments

comparable values are 74 and 67 Å, respectively (see Rand et al., 1975; Church et al., 1986). Also, the lamellar long spacings of those lipids increase at T_m (see Fig. 8 and Table 4), in marked contrast to straight chain PCs such as DPPC for which the chain-melting phase transition is accompanied by a decrease in the lamellar repeat distance (for examples, see Rand et al., 1975; Church et al., 1986). In principle the above observations could be the result of one or more of the following: 1) significant swelling of the interlamellar water layers at T_m, 2) strong tilting of the hydrocarbon chains at temperatures below T_m, and 3) hydrocarbon chain interdigitation at temperatures below T_m. Of these possibilities, significant tilting of the hydrocarbon chains below T_m can be eliminated because the characteristic pattern of wide angle reflections near 4.2 Å (see Tardieu et al., 1973) are not observed in the x-ray diffraction experiment (data not shown). Other possibilities are that the di-(*dl*)-2-alkylalkanoyl PCs form gel and liquid-crystalline phases that are less hydrated than are those of the corresponding unsubstituted straight-chain lipids and/or that some form of hydrocarbon chain interdigitation exists in the gel phases of the (*dl*)-2-alkyl-alkanoyl PCs. It should be noted, however, that the gel-phase small angle diffraction pattern typically observed (see Fig. 9) is more typical of noninterdigitated gel phases.

TABLE 4 Lamellar long spacings of selected di-(*dl*)-2-alkyl-hexadecanoyl PCs

2-Alkyl Substituent	* Lamellar Long Spacing (Å)	
	L_β Phase	L_α Phase
-Butyl	51.9 (20°C)	67.0 (30°C)
-Benzyl	51.5 (15°C)	60.2 (30°C)
-(2-methyl-butyl)	54.5 (15°C)	58.2 (20°C)
-(3-methyl-butyl)	55.4 (15°C)	58.2 (20°C)
-(3,3-dimethyl butyl)	49.0 (10°C)	‡na
-(cyclohexyl methyl)	53.58 (5°C)	‡na

* Lamellar long spacings (Å) were measured at the temperatures indicated in brackets.
‡Not Applicable. Stable L_α phases were not observed.

The lamellar phases of the lipids examined become unstable at temperatures above T_m and transform to structures with markedly different diffraction characteristics. For lipids such as di-(*dl*)-2-(3, 3 dimethyl butyl) hexadecanoyl PCs and di-(*dl*)-2-(cyclohexyl-methyl) hexadecanoyl PC, diffraction patterns indicative of lamellar phases are only observed at temperatures below T_m and are typified by that shown Fig. 9 (*top panel*), a diffraction pattern observed in the gel phase of these lipids. At temperatures above T_m both di-(*dl*)-2-(3, 3 dimethyl butyl) hexadecanoyl PCs and di-(*dl*)-2-(cyclohexyl-methyl) hexadecanoyl PC exhibit diffraction patterns characterized by peak distances in the ratio $1:\sqrt{3}:\sqrt{4}:\sqrt{7}$ as shown in Fig. 10 *A*. Also, the lattice constant remains fixed even in the presence of excess water and decreases smoothly as the temperature increases. The inverted nature of this phase was demonstrated by the fact that the dispersions

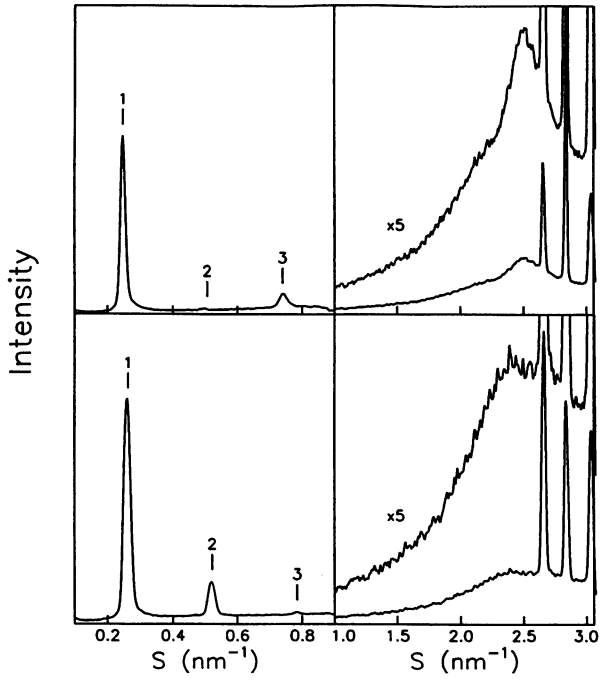

FIGURE 9 Radially integrated x-ray powder diffraction patterns exhibited by the lamellar phases of selected di-(*dl*)-2-alkyl-alkanoyl-hexadecanoyl PCs. Diffraction patterns are presented for the lamellar gel phase of di-(*dl*)-2-(cyclohexyl methyl) hexadecanoyl PC (*top*) and the lamellar liquid-crystalline phase of di-(*dl*)-2-benzyl dodecanoyl PC (*bottom*) at −30°C. Small angle diffraction patterns are shown on the left, and wide angle diffraction patterns are shown on the right. In the wide angle diffraction patterns the sharp peaks between 2.5 and 3.0 Å are the result of ice crystals.

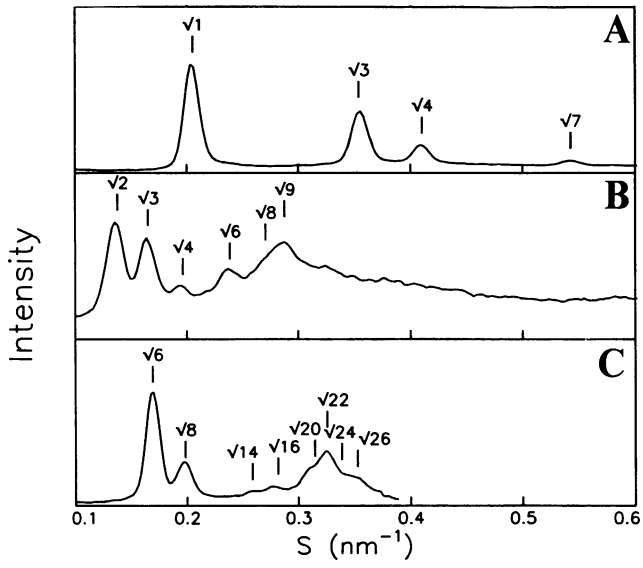

FIGURE 10 Radially integrated x-ray powder diffraction patterns exhibited by the inverted nonlamellar phases of selected di-(*dl*)-2-alkyl-hexadecanoyl PCs. Diffraction patterns are presented for: (*A*) The inverted hexagonal phase, di-(*dl*)-2-(cyclohexyl methyl)-hexadecanoyl PC at 85°C; (*B*) The cubic Pn3m/Pn3 phase, di-(*dl*)-2-(2-methyl butyl)-hexadecanoyl PC at 85°C; and (*C*) The gyroid Ia3d phase, di-(*dl*)-2-(2-methyl butyl)-hexadecanoyl PC at 20°C.

swelled to a limited degree in excess water. The above features clearly indicate that both di-(*dl*)-2-(3, 3 dimethyl butyl) hexadecanoyl PCs and di-(*dl*)-2-(cyclohexyl-methyl) hexadecanoyl PC form H_{II} phases at temperatures above T_m, thus confirming the conclusions drawn from the ^{31}P-NMR spectroscopic data presented above.

Small angle x-ray diffraction patterns of di-(*dl*)-2-(3-methyl butyl) hexadecanoyl PC and di-(*dl*)-2-(2-methyl butyl) hexadecanoyl PC were also acquired to determine the structure of the isotropic phases formed by some of these lipids. These lipids are lamellar at temperatures below T_m, their chain-melting phase transitions are accompanied by an expansion of their respective lamellar lattices (see Fig. 8), and, at temperatures well above T_m, they exhibit complex nonlamellar diffraction patterns. As was observed in the ^{31}P-NMR spectroscopic experiment, di-(*dl*)-2-(2-methyl butyl) hexadecanoyl PC exhibits a very complex phase behavior when heated to temperatures well above T_m. When heated to temperatures between 40 and 50°C, the lamellar diffraction pattern changes to the form shown in Fig. 10 *C*. This lipid exhibits relatively diffuse diffraction peaks in this phase but enough peaks are resolved to enable the assignment of the latter to the gyroid phase (space group Ia3d). Upon heating to very high temperatures, the diffraction pattern exhibited by this lipid changes to a form similar to that shown in Fig. 10 *B*. The change in diffraction pattern is a correlated with the abrupt decrease in the spacings observed at high temperature (see Fig. 8). This phase exhibits better resolved diffraction peaks, which are consistent with a phase of cubic symmetry belonging to the space groups Pn3m or Pn3 (see Fig. 10 *B*). Thus, as was inferred from the ^{31}P-NMR spectroscopic study, this lipid seems to form two structurally distinct nonlamellar cubic phases. Also, consistent with the ^{31}P-NMR spectroscopic data, the cubic phase that forms at high temperature does not revert to the low temperature form upon cooling. Thus, upon cooling the Pn3m/Pn3 cubic phase persists to temperatures near 10°C when the lamellar gel phase reappears. Diffraction patterns consistent with the formation of a Pn3m/Pn3 cubic phase (see Fig. 10 *B*) were also observed when samples of di-(*dl*)-2-(3-methyl butyl)-hexadecanoyl PC were heated to temperatures above 50°C. Thus, at least with the representative lipids studied here, the x-ray diffraction data is consistent with the general phase assignments made by ^{31}P-NMR spectroscopy.

DISCUSSION

We have demonstrated that the substitution of modestly sized hydrophobic groups at C2 of the fatty acyl chains induces nominally bilayer-preferring PCs to form inverted nonlamellar structures, a possibility suggested in previous studies of branched chain diacyl PCs (Nuhn et al., 1986; Brezesinski et al., 1987). Hydrophobic substituents near the bilayer polar/apolar interface probably destabilize the lamellar phases of these lipids by perturbing lateral interactions between the hydrocarbon chains. In the gel phase, the disruptive influence of such groups is manifested primarily by a lowering of the

gel/liquid-crystalline phase temperature. Our x-ray diffraction studies also indicate that the lamellar gel phases formed by these di-(*dl*)-2-alkyl-alkanoyl PCs differ significantly from those formed by saturated straight-chain PCs. Specifically, these lipids seem to form poorly hydrated bilayer arrays at temperatures below 0°C, their lamellar repeat distances are smaller than those of the unsubstituted analogue, and their hydrocarbon chains may be interdigitated at temperatures below T_m. However, our DSC studies indicate that the enthalpy change occurring at the chain-melting phase transitions of the majority of the lipids studied is comparable to, and sometimes exceeds that of, the unsubstituted analogue (see Table 2). Therefore the destabilization of hydrocarbon chain interactions evidenced by the diminution of the T_m's of these di-(*dl*)-2-alkyl-alkanoyl PCs are not reflected in the enthalpy changes coincident with the chain-melting phase transition. Thus, the nature of the structural changes that occur at their chain-melting phase transitions must differ from those of the unsubstituted analogue.

In the chain-melted state, the destabilization of the lamellar phase by the various substituents is manifest by the appearance of inverted nonlamellar phases. The tendency for these lipids to form nonlamellar phases generally increases with increases in the length, volume and rigidity of the 2-alkyl substituents. With the smaller substituent groups cubic phases are the only nonlamellar structures formed, whereas with the larger substituent groups inverted hexagonal phases are also formed. However, we did find some interesting exceptions to the above generalization. For instance, the methyl group destabilizes the lamellar gel and liquid-crystalline phases to a greater extent than do the larger ethyl or propyl groups. Also, whereas the benzyl and cyclohexyl-methyl groups are roughly comparable in size, the aromatic group is considerably less effective at promoting nonlamellar phases than is its alicyclic counterpart. Indeed the ^{31}P-NMR spectroscopic data also suggest that the methyl substituent may well be a more potent promoter of nonlamellar phases than is the considerably larger benzyl group. It is therefore difficult to reconcile all of our observations strictly on the basis of the sizes of the substituents, if the glycerol backbone retains its usual conformation (perpendicular to the bilayer surface) when large hydrophobic substituents are placed at C2 of the fatty acyl chains. This is because the lipid molecules cannot assume an energetically favorable packing mode in which there are close van der Waals contacts between the hydrocarbon chains without the large bulky hydrophobic substituent at C2 of the *sn*2 fatty acyl chain projecting into the hydrated portion of the lipid bilayer. Interestingly, if the glycerol backbone were arranged parallel to the bilayer surface, relatively close van der Waals contacts between the hydrocarbon chains should be possible without the placement the 2-alkyl substituents (both the *sn*1 and *sn*2) into a polar environment. However, such a conformational change does incur a significant energetic cost and will probably occur only when the hydrophobic substituents become sufficiently large. In principle, the differential effect suggested above could explain many of the properties of these PCs. For example, a change in the conformation of the glycerol backbone may account for the discontinuity observed in our studies of the effect of increasing the length of the 2-alkyl substituent. Moreover, the same differential effect could also explain why a benzyl group is considerably less effective at promoting the formation of a nonlamellar phase than is the cyclohexyl methyl group. This is because the aqueous solubility of benzene (1 part in 1430) is several orders of magnitude greater than that of cyclohexane, and as a result the location of a benzene ring in a hydrated environment will be less energetically costly than is the case with a cyclohexane ring. Consequently, it may be energetically favorable for the benzyl-substituted lipid to retain the normal conformation of its glycerol backbone, whereas this would not be the case for the cyclohexyl-methyl analogue. Interestingly, the proposed change in the conformation of the glycerol backbone may also promote the formation of chain-interdigitated structures at temperatures below T_m. This is because such a conformational change would result in a lateral expansion of the hydrophobic domain of the lipid bilayer. Such expansion will facilitate the accommodation of the hydrophobic substituents between the hydrocarbon chains, but it will also increase the mean separation of the main fatty acyl chains thereby increasing the probability of forming chain-interdigitated structures. Under such conditions the latter would be favored in preference to subgel phases when these lipids are incubated at low temperatures.

Another unusual feature of the thermotropic phase behavior of these di-(*dl*)-2-alkyl-alkanoyl PCs is the fact that the absolute temperatures at which the lamellar phases of these lipids become unstable with respect to a nonlamellar phase increases with increases in the length of the main acyl chain (see Fig. 7). The chain-length dependence described above is the opposite of that exhibited by most non-lamella-forming lipids and is not predicted from theoretical considerations based on either lipid shape (Israelachvilli et al., 1977, 1980) or spontaneous monolayer curvature (see Kirk et al., 1984; Gruner et al., 1985). Presumably, it can be attributed to chain-length-dependent alterations of the lateral stress profiles within the monolayers formed by these lipids (see below).

Studies of this new class of non-lamella-forming PCs may also provide insight into the relationship between lamellar/nonlamellar phase behavior and the lateral stress profile within a lipid monolayer (see Helfrich, 1980; Petrov and Bivas, 1984; Seddon, 1990). The stress profile, S(z), specifies the sign and magnitude of stress parallel to the membrane surface at a given depth, z, along a line perpendicular to the surface of the monolayer, as is schematically represented in Fig. 11. In several of the more commonly accepted representations, the profile is dominated by three regions: a repulsive interaction due to the headgroups; a cohesive interfacial tension in the hydrophylic/hydrophobic contact region; and a repulsive hydrocarbon chain region (see the regions labeled H, I, and C in Fig. 11). Indeed, knowledge of the lateral stress profile could reveal a great deal about forces active in a lipid monolayer. For example, if it can be assumed that the net stress is zero and reasonable constraints are

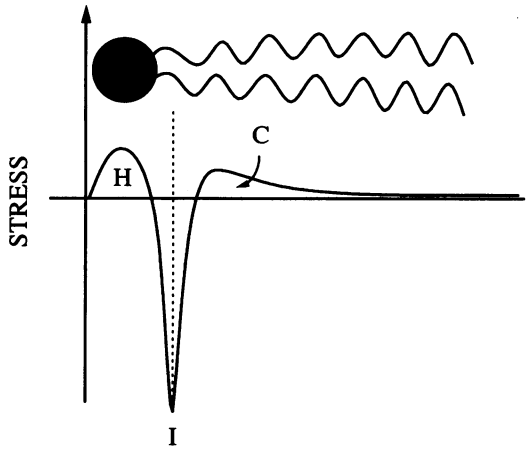

FIGURE 11 Diagrammatic representation of the "lateral stress profile" of a melted lipid monolayer. "I" represents interfacial tension, whereas "H" and "C" represent the stress profiles in the headgroup and acyl chain regions, respectively.

placed on "local flatness," then the integral across the monolayer of the first moment of the stress profile would yield the product of monolayer rigidity (i.e., stiffness) and spontaneous curvature. Thus, in so far as the stiffness is nearly constant for monolayers of the same thickness, the integral of the first moment of the stress profile would yield the spontaneous monolayer curvature and, hence, the tendency for the system to adopt nonlamellar phases. In this respect the stress profile captures a more exact description of the system than is encoded, for example, by either single-valued shape parameters (see Israelachvilli et al., 1977, 1980) or the spontaneous monolayer curvature (see Kirk et al., 1984; Gruner et al., 1985) alone. Unfortunately, at this time there is no known way to directly measure S(z). In comparing these new nonlamella-forming PCs with their unsubstituted bilayer-preferring counterparts, it is tempting to assume that the dominant effect on S(z) is on the interfacial "I" region of Fig. 11. If this should prove to be true, then these new classes of compounds would be very useful as probes to the "I" component of S(z).

Our data also highlight the potential usefulness of these lipids in studies of the structure of nonlamellar phases as well as the mechanism of lamellar/nonlamellar phase transitions. It has been postulated for some time that cubic phases may be crucial intermediates en route to the formation of an inverted hexagonal phase (see Siegel, 1986a, b, and references cited therein), and that kinetically hindered cubic phases may exist between lamellar and inverse hexagonal phases (Shyamsunder et al., 1988; Andersen et al., 1988). These hypotheses are supported by our data. We have also shown that a number of different types of cubic phases can exist, not all of which may be structural intermediates from which an H_{II} phase can be easily formed. This point is clearly demonstrated by our studies of di-(*dl*)-2-(3, 3-dimethyl-butyl)-hexadecanoyl PC and di-(*dl*)-2-(2-ethyl-butyl)-hexadecanoyl PC, and has also been inferred from the work of Shyamsunder et al. (1988). We have also shown that one can produce diacyl PCs for which various cubic phases are stable in an experimentally accessible temperature range by an appropriate choice of main acyl chain length and substituent group. This is significant because many non-lamella-forming lipids do not form homogeneous stable cubic phases or do so only after a fairly tedious protocol (see, for example, Shyamsunder et al., 1988). The availability of these new types of non-lamella-forming lipid species and the facility to design lipid molecules that form specific types of nonlamellar phases now make possible more definitive studies of the structures of such phases and their conversion to other lamellar and nonlamellar structures. Also, although comparable studies on lipids with other nominally lamella-forming polar headgroups (e.g., phosphatidylserine, phosphatidylglyerol, and diglucosyldiacylglycerol) have not yet been done, it may be possible to produce non-lamella-forming analogues of those lipids as well. With such molecules it should be feasible to design experiments that require fine control over the "non-lamella-forming tendencies" of a lipid bilayer using almost any lipid polar headgroup. Such a facility may be especially useful in studies of the effect of non-lamella-forming lipids on the biological activity of intrinsic or other membrane-associated proteins. The ability to dramatically alter the non-lamella-forming potential of a lipid bilayer without introducing any variations in polar headgroup composition should simplify the design of such experiments and the interpretation of the ensuing results.

Finally, the activity of protein kinase C was recently shown to be increased by diacyl PCs with hydrophobic groups near to the bilayer polar/apolar interface (Charp et al., 1988). These results are very interesting given our demonstration that the substitution of hydrophobic groups near the polar/apolar interfacial region of the lipid bilayer promotes the formation of nonlamellar phases and previous demonstrations that protein kinase C is activated by non-lamella-forming lipids (see Epand and Bottega, 1988; Epand et al., 1988). Because the possibility that the lipids used by Charp et al., (1988) may have an enhanced non-lamella-forming potential was not recognized by the authors, it would be very interesting if their results were re-evaluated with due consideration to the findings presented here.

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