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A Differential Scanning Calorimetric Study of the Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing Linear Saturated Fatty Acyl Chains†

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ABSTRACT: The thermotropic phase behavior of a series of 1,2-diacylphosphatidylcholines containing linear saturated acyl chains of 10–22 carbons was studied by differential scanning calorimetry. When fully hydrated and thoroughly equilibrated by prolonged incubation at appropriate low temperatures, all of the compounds studied form an apparently stable subgel phase (the L_c phase). The formation of the stable L_c phase is a complex process which apparently proceeds via a number of metastable intermediates after being nucleated by incubation at appropriate low temperatures. The process of L_c phase formation is subject to considerable hysteresis, and our observations indicate that the kinetic limitations become more severe as the length of the acyl chain increases. The kinetics of L_c phase formation also depend upon whether the acyl chains contain an odd or an even number of carbon atoms. The L_c phase is unstable at higher temperatures and upon heating converts to the so-called liquid-crystalline state (the L_α phase). The conversion from the stable L_c to the L_α phase can be a direct, albeit a multistage process, as observed with very short chain phosphatidylcholines, or one or more stable gel states may exist between the L_c and L_α states. For the longer chain compounds, conversions from one stable gel phase to another become separated on the temperature scale, so that discrete subtransition, pretransition, and gel/liquid-crystalline phase transition events are observed. We have also examined some aspects of the hysteresis in the pretransition, and our observations indicate that the behavior can be approximated by a reversible process which is subject to some modest kinetic limitations, these limitations again becoming more severe for the longer chain phosphatidylcholines.

Lipid bilayers composed of phosphatidylcholines (PCs)[1] containing two identical linear saturated fatty acyl chains are the most thoroughly studied of the model membrane systems. Such studies have employed a variety of physical techniques and have tended to concentrate on a few members of the homologous series, with the result that the thermotropic phase behavior of some of these PCs (especially DPPC) is relatively well understood. From the extensive literature on these compounds, it is expected that when fully hydrated and thoroughly equilibrated by prolonged incubation at low temperatures, the longer chain PCs like DPPC ($n \geq 16$) form a highly ordered, condensed, crystallike phase (the L_c phase) in which the hydrocarbon chains are in a fully extended, all-trans conformation (Cameron & Mantsch, 1982) with their long axes tilted to the bilayer normal (Ruocco & Shipley, 1982). In that phase, the phosphate head groups are relatively immobile (Fuldner, 1981; Lewis et al., 1984) and are assumed to lie parallel to the bilayer plane (Pearson & Pascher, 1979; Griffin et al., 1978), and the interfacial region of the lipid bilayer is partially dehydrated (Camerson & Mantsch, 1982). Upon heating, the L_c phase

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[1] Abbreviations: DSC, differential scanning calorimetry; DTA, differential thermal analysis; NMR, nuclear magnetic resonance; PC, phosphatidylcholine (in this paper, a phosphatidylcholine is usually designated by the notation *n*:0-PC, where *n* is the number of carbon atoms per acyl chain with the zero indicating the absence of double bonds); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; HS-DSC, high-sensitivity differential scanning calorimetry.

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undergoes a transition (the subtransition) to the $L_{\beta'}$ phase in which there is an increased mobility of the phosphate head group (Fuldner, 1981; Lewis et al., 1984), a greater penetration of water into the interfacial region of the bilayer, and a considerable reduction in the tilt of the acyl chains to the bilayer normal (Ruocco & Shipley, 1982). Further heating of the $L_{\beta'}$ phase results in a progressive increase in the mobility of the phosphate head group and eventually a conversion (the pretransition) to the $P_{\beta'}$ phase. The $P_{\beta'}$ phase is characterized by the absence of any hydrocarbon chain tilt to the bilayer normal (Rand et al., 1975) and the onset of rigid-body rotation of the lipid molecules along their long axes (Boroske & Trahms, 1983; Trahms et al., 1983), as well as the appearance of "ripples" on the bilayer surface [see Doniah (1979) and Janiak et al. (1976)]. Further heating of the $P_{\beta'}$ phase induces the cooperative melting of the acyl chains (the gel/liquid-crystalline phase transition) to form the L_α phase in which there is the onset of trans–gauche isomerism in the acyl chains (Mendelsohn & Mantsch, 1986), fast lateral diffusion of the lipid molecules (Träuble & Sackmann, 1972; Vaz et al., 1984), and fast axially symmetric motion of the phosphate head group (Fuldner, 1981; Seelig, 1978).

DSC has been the technique of choice for obtaining basic thermodynamic data on the phase behavior of the *n*-acyl-PCs and many other lipid molecules [see McElhaney (1982)]. In the case of the *n*-acyl-PCs, the bulk of the calorimetric data was acquired before techniques for preparing highly purified PCs were developed and before high-sensitivity calorimeters were generally available. These factors almost certainly contributed to the considerable "scatter" in the thermodynamic data reported in the literature [see Silvius (1982) and Small (1986)]. Furthermore, a large amount of data was collected prior to 1980, at which time the ability of these compounds to form the L_c phase was first reported (Chen et al., 1980). It is also apparent from a survey of the literature that there have been relatively few studies on the phase properties of *n*-acyl-PCs whose hydrocarbon chains contain an odd number of carbon atoms. The relative shortage of such data has assumed greater significance since recent studies have demonstrated that the thermotropic phase properties of lipids can be markedly dependent on whether their acyl chains contain an odd or an even number of carbon atoms (Lewis & McElhaney, 1985a,b; Mantsch et al., 1985; Church et al., 1986; Yang et al., 1986). Those studies have also shown that, in a homologous series of PCs, there can be marked behavioral discontinuities between the shorter chain compounds and their longer chain counterparts. That this may also be true of the *n*-acyl-PCs was suggested by a recent report (Finegold & Singer, 1986) which indicated that, while some shorter chain homologues also exhibit gel-state polymorphism, the details of their thermotropic phase behavior may differ from their longer chain counterparts. In an attempt to clarify some of the above issues and to fill in some of the obvious gaps in the literature, we have undertaken a systematic study of the thermotropic phase behavior of the homologous series of *n*-acyl-PCs with acyl chains ranging from 10 to 22 carbon atoms. In this study, we have attempted to ensure a consistently high purity of all the PC samples examined and to explore the full extent of the polymorphic gel phase behavior of these compounds.

Materials and Methods

The phosphatidylcholines used in this study were synthesized from their respective fatty acids by methods which have been shown to yield highly purified samples (Lewis & McElhaney, 1985a). To ensure consistency of purity and acyl chain homogeneity, the fatty acids used for the synthesis were themselves synthesized from appropriate precursors by methods designed to ensure acyl chain homogeneity [see Lewis and McElhaney (1985a)]. The high-sensitivity differential scanning calorimetric studies were performed in Microcal MC-1 and MC-2 high-sensitivity calorimeters, while the low-sensitivity DSC studies were performed in a Perkin-Elmer DSC-2C instrument equipped with a thermal analysis data station. Unless otherwise stated, DSC measurements were all made at the slowest scan rates feasible with the instruments (Microcal MC-1, $\simeq$5–6 °C h^{-1}; Microcal MC-2, 11–12 °C h^{-1}; Perkin-Elmer DSC-2C, 18.75 °C h^{-1}). The methods for sample preparation and quantification were the same as previously used in this laboratory (Lewis & McElhaney, 1985a). To ensure equilibration between the $L_{\beta'}$ and $P_{\beta'}$ phases, the samples were all slowly cooled through the range of the pretransition temperatures prior to data acquisition in the heating mode.

Results

Thermotropic Phase Behavior of Nonannealed Samples. DSC thermograms of aqueous dispersions of the 1,2-di-*n*-acyl-PCs studied are shown in Figures 1 and 2. The thermograms shown were recorded by high-sensitivity DSC (Figure 1) and low-sensitivity DSC (Figure 2) before the samples were equilibrated by prolonged annealing at low temperatures. As expected, all of the PCs studied exhibit at least one major, highly cooperative thermotropic event, at temperatures which increased as the length of the acyl chains increased. In addition, all of the PCs with acyl chains of 13 or more carbon atoms (except 22:0-PC) exhibited another broad thermotropic event at a temperature lower than that of the main endothermic transition. This type of behavior has been well documented for the 1,2-di-*n*-acyl-PCs with acyl chains of 14–20 carbon atoms [see Silvius (1982) and Small (1986) and references cited therein], and the events have been assigned to the well-characterized pretransition (lower temperature event) and gel/liquid-crystalline phase transition (higher temperature event), respectively.

The high-sensitivity DSC thermograms (Figure 1) show that the gel/liquid-crystalline phase transitions of the longer chain PCs ($n \geq 13$) are highly cooperative thermotropic events. For the samples used in this study, the transition widths (measured at half of the height of the heating endotherm) increase with increasing acyl chain length and range from values near 0.1 °C ($n = 13$) to values near 0.5 °C ($n = 22$, see Table IV). For some of these PCs ($n = 13$–16), an accurate measurement of their transition widths was compromised by the resolution limits of the Microcal MC-1 instrument. Nevertheless, the values listed in Table IV, though nominally higher than that reported for a highly purified DPPC sample (Albon & Sturtevant, 1978), testify to the high purity of the samples used in this study. A close inspection of Figure 1 also shows that there are distinct high-temperature shoulders on the gel/liquid-crystalline phase transition endotherms of ditridecanoyl-PC ($n = 13$) and dipentadecanoyl-PC ($n = 15$). These high-temperature shoulders are not observed with any of the other PCs examined here but have previously been observed in some DTA studies on ditridecanoyl-PC (Silvius et al., 1979). The physical basis of this behavior is currently unknown.

The DSC thermograms shown in Figure 2 indicate that there was little or no hysteresis in the gel/liquid-crystalline phase transitions of the longer chain PCs ($n \geq 13$), since they are generally observed at the same temperatures (±0.3 °C) in both the heating and cooling modes. The peak temperatures of the exothermic transition observed upon cooling of the

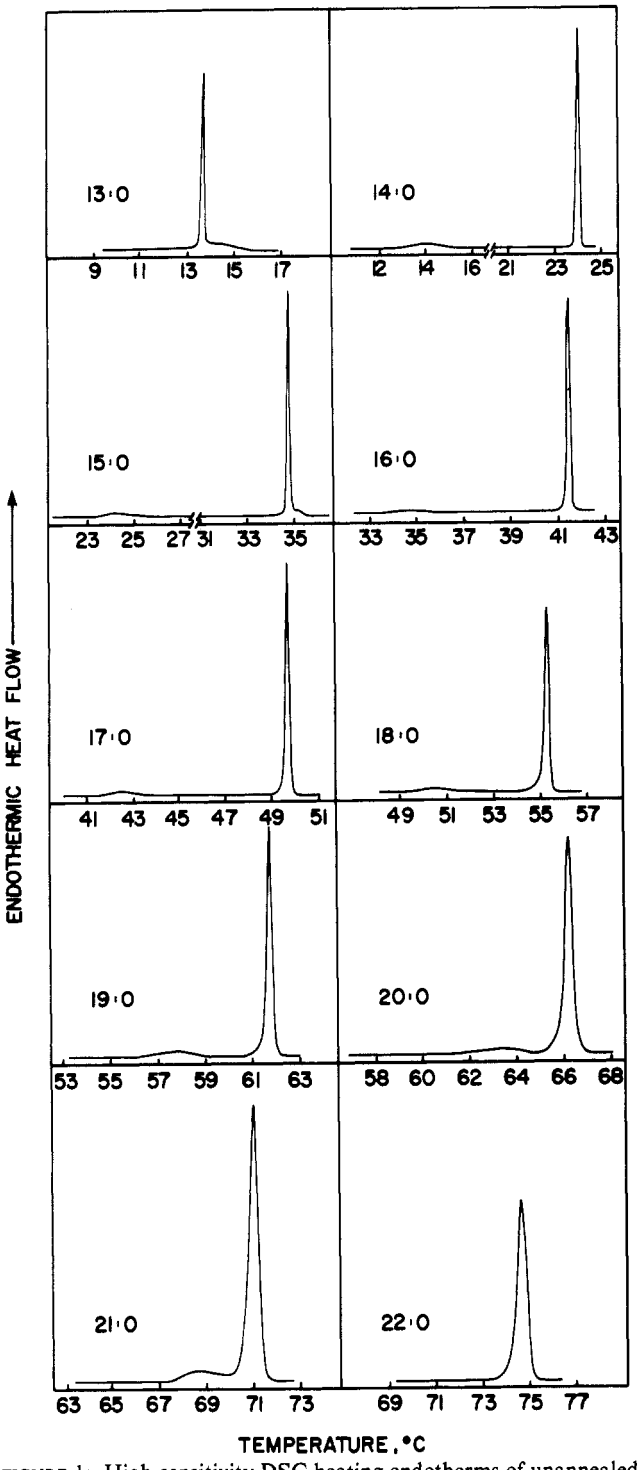

FIGURE 1: High-sensitivity DSC heating endotherms of unannealed fully hydrated samples of 1,2-di-*n*-acyl-PCs (n = 13–22). Scan rate = 5–6 °C·h^{-1}.

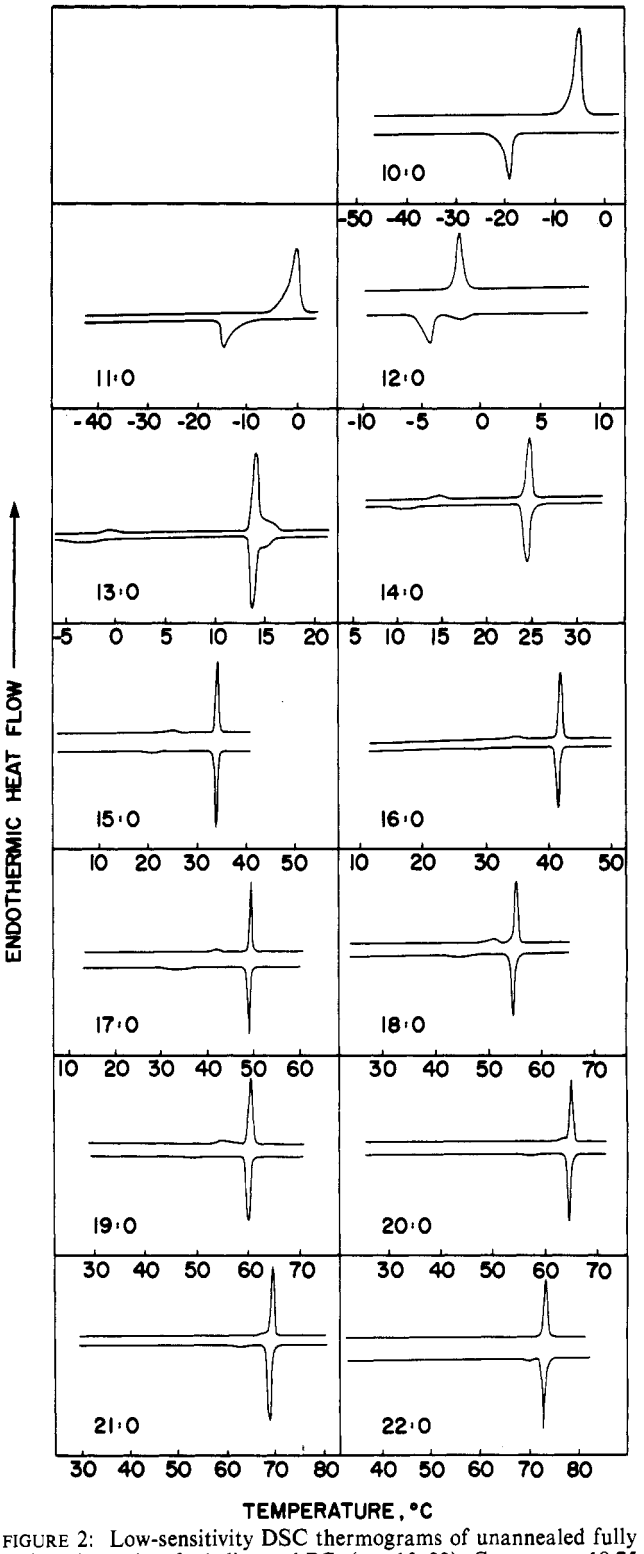

FIGURE 2: Low-sensitivity DSC thermograms of unannealed fully hydrated samples of 1,2-di-*n*-acyl-PCs (n = 10–22). Scan rate = 18.75 °C·h^{-1}.

short-chain PCs ($n \leq 12$) are clearly lower than those of their heating endotherms. This effect was more pronounced with didecanoyl-PC and diundecanoyl-PC, and unlike didodecanoyl-PC, it was not attributable to classical hysteresis. For those PCs (10:0-PC and 11:0-PC), the observed thermotropic behavior is dependent primarily upon the thermal history of the sample and is unaffected by prolonged annealing at temperatures between those of the heating and cooling peaks. This type of behavior has been correlated with seemingly direct interconversions between the L_c and L_α phases of some other PCs (Lewis & McElhaney, 1985a,b), and our unpublished ^{31}P NMR spectroscopic data have shown that a similar process is occurring with the short chain PCs (n = 10, 11) studied here.

An increase in acyl chain length results in a progressive increase in the characteristic temperatures of the pretransition and gel/liquid-crystalline phase transitions and a decreased separation between both events on the temperature scale (see Figure 9 and Table II), as expected from previous studies [see Silvius (1982) and Small (1986)]. It is also apparent from Figure 2 that the calorimetric observation of the pretransition is more difficult in the cooling mode. For some of the compounds, the process is barely calorimetrically observable in the cooling mode, and in all cases, the cooling exotherms are considerably broader than the corresponding heating endo-

Table I: Low-Temperature Incubation Conditions Used To Induce Formation of L_c Phases of 1,2-Di-*n*-acylphosphatidylcholines

PC	conditions
10:0	cool sample slowly to −50 °C, warm to −25 °C, and incubate at that temp for 30 min
11:0	cool sample slowly to −50 °C, warm to −15 °C, and incubate at that temp for 30 min
12:0 + 13:0	cool sample slowly to −40 °C, incubate at −25 °C overnight, then transfer to an ice/water bath, until bulk aqueous phase melts (3–5 days), and then incubate at 0 °C for 5 days
14:0	cool sample slowly to −40 °C and incubate at that temp for 1 h; warm sample slowly to −10 °C and then incubate at that temp for 1 week; transfer sample to an ice bath until bulk aqueous phase melts and then incubate at 0 °C for 1 week
15:0	cool sample slowly to −10 °C and incubate at that temp for 1 week; transfer sample to an ice bath until bulk aqueous phase melts and then incubate at 0 °C for 1 week
16:0–22:0	cool samples slowly through the range of their pretransition temp and then incubate at 0–4 °C for periods ranging from 6 days to 6 weeks (16:0-PC), or until the phase forms (≅16 months for 21:0-PC)

Table II: Calorimetrically Determined Heating Endothermic Transition Temperatures of Aqueous Dispersions of 1,2-Di-*n*-acylphosphatidylcholines

	calorimetrically obsd transition temp[a] (°C)					
PC	$L_c \rightarrow L_\alpha$[b]	$L_c \rightarrow L_{\beta'}$[b]	$L_c \rightarrow P_{\beta'}$[b]	$L_{\beta'} \rightarrow P_{\beta'}$[c]	$L_{\beta'} \rightarrow L_\alpha$	$P_{\beta'} \rightarrow L_\alpha$[d]
10:0	−5.7					
11:0	−0.8					
12:0	7.0					−2.1
13:0	14.4		11.7	−0.8		13.7
14:0			16.2	14.3		23.9
15:0		22.3		24.8		34.7
16:0		21.2		34.2		41.4
17:0		25.8		43.0		49.8
18:0		28.2		50.7		55.3
19:0		33.0		57.8		61.8
20:0		37.8		63.7		66.4
21:0		28.0		68.7		71.1
22:0		32.1			74.8	

[a] These are the calorimetrically determined transition temperatures. Since many of the processes are kinetically limited, they may not be the true equilibrium transition temperatures for the observed event. [b] In cases where more than one L_c phase was observed the $L_C \rightarrow$?? transition temperature refers to that of the most stable L_c phase observed. [c] Pretransition temperatures: these were determined at the slowest rate feasible with the instrument used (18.75 °C·h^{-1} for 13:0-PC, 5–6 °C·h^{-1} for all other PCs). [d] Gel/liquid-crystalline phase transition temperatures.

therms and are observed at lower temperatures. Furthermore, the calorimetrically determined transition temperatures (both heating and cooling) are dependent upon the scan rate. The above is directly attributable to a modest hysteresis in the interconversions between the $L_{\beta'}$ and $P_{\beta'}$ phases of these PCs [see Silvius (1982) and references cited therein]. A close inspection of the cooling exotherm of 22:0-PC (see Figure 2) shows that there is a small exothermic event occurring at temperatures lower than that of the main cooling exotherm. Given the hysteresis in the interconversion between the $L_{\beta'}$ and $P_{\beta'}$ phases, this observation provides some direct experimental evidence that the single heating endotherm characteristic of a nonannealed sample of 22:0-PC probably incorporates both the pretransition and gel/liquid-crystalline phase transition events.

Thermotropic Phase Behavior of Annealed Samples. Chen et al. (1980) demonstrated that a prolonged incubation of three of these PCs (n = 16–18) at temperatures around 0–4 °C resulted in a slow conversion of the $L_{\beta'}$ phase to the so-called subgel phase (L_c phase). We have tried various regimes of low-temperature incubation so as to determine whether all of the PCs used in this study can form L_c phases and to obtain some insight into the conditions required for their formation. Some initial heating endotherms obtained after suitable annealing (see Table I) of these PCs are shown in Figure 3. For the shorter chain PCs (n = 10, 11), the DSC heating endotherms are indistinguishable from those obtained without extensive annealing (see Figure 2) and do not change if annealed

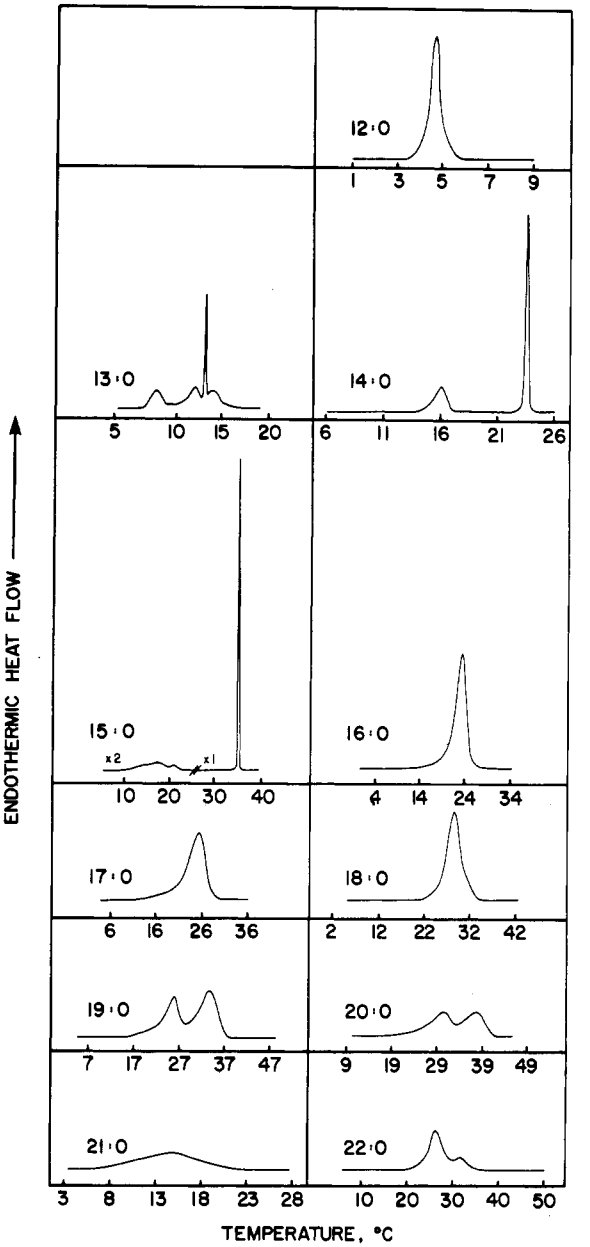

FIGURE 3: High-sensitivity DSC heating endotherms of fully hydrated samples of 1,2-di-*n*-acyl-PCs (n = 12–22) after prolonged annealing using appropriate low-temperature regimes (scan rate = 11.6 °C·h^{-1}). The pretransition and gel/liquid-crystalline phase transition endotherms of the longer chain PCs ($n \geq 16$) are not shown in this figure.

for considerably longer periods. Our unpublished ^{31}P NMR spectroscopic data and the magnitude of the enthalpy changes associated with those thermotropic events (see Table III) suggest that the short-chain PCs did form subgel phases under our experimental conditions. Thus, the single heating endotherms exhibited by 10:0-PC and 11:0-PC are most probably the result of net $L_c \rightarrow L_\alpha$ conversions.

Table III: Enthalpy Changes Associated with Thermotropic Phase Transitions of Aqueous Dispersions of 1,2-Di-*n*-acylphosphatidylcholines

PC	enthalpy change (kcal·mol^{-1})					
	$L_c \rightarrow L_\alpha$[a]	$L_c \rightarrow L_{\beta'}$[a]	$L_c \rightarrow P_{\beta'}$[a]	$L_{\beta'} \rightarrow P_{\beta'}$	$L_{\beta'} \rightarrow L_\alpha$	$P_{\beta'} \rightarrow L_\alpha$
10:0	18.3					
11:0	9.5					
12:0	13.4					1.8
13:0	12.3			0.5		4.4
14:0			6.2	1.1		5.9
15:0		6.3		0.9		6.9
16:0		6.2		1.1		7.7
17:0		6.5		1.1		8.7
18:0		6.7		1.2		9.8[b]
19:0		6.9		1.3		10.7[b]
20:0		4.9		1.4		11.4[b]
21:0		2.9		1.4		12.2[b]
22:0		4.3			14.9	

[a] The formation of stable L_c phases in these PCs is a complex process which is believed to proceed via at least two intermediate L_c phases. Furthermore, the kinetics of formation of those phases are slow, and in the case of the longer chain compounds, the process of formation was not complete after a 2-year period. Thus, for all transitions involving a transformation of an L_c phase, the enthalpy change observed is the maximum that has been obtained under our conditions and must be regarded as the lower limit of the enthalpy change associated with these given process(es). [b] There was some (<1%) hydrolysis of the samples during the DSC experiment. The quoted enthalpy values are probably slightly underestimated.

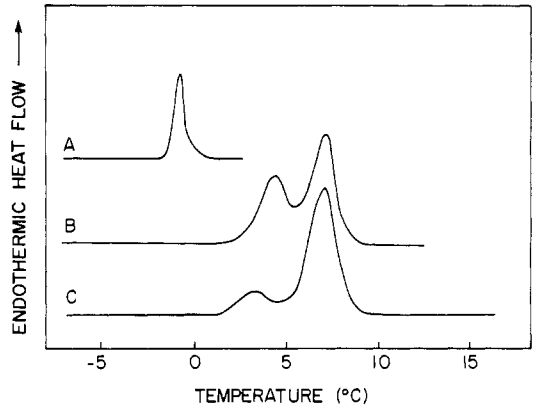

FIGURE 4: Initial DSC heating thermograms of 12:0-PC after various periods of annealing at 0 °C (scan rate = 11.6 °C·h^{-1}). The three thermograms are not drawn on the same y axis. (A) No annealing; (B) 1 day annealing; (C) 3 days annealing.

It is also apparent from Figure 3 that, once equilibrated, all of the other PCs studied showed evidence of thermotropic events in addition to those shown in Figures 1 and 2 and that the thermotropic phase behavior associated with the so-called "subtransition" is more complex than first reported by Chen et al. (1980). This is described in detail below.

(*A*) *Didodecanoyl-PC.* The equilibration of 12:0-PC under the conditions described in Table I is accompanied by a complete suppression of all thermotropic events at temperatures around −2 °C and the appearance of two broad endotherms of considerably greater enthalpy with maxima at 3 and 4.5 °C (scan rate 11.6 °C h^{-1}). With prolonged incubation at 0 °C, there appears to be some growth of the high-temperature peak at the expense of the low-temperature peak (see Figure 4) and a significant increase in the measured total enthalpy of the transitions (see Table III). The conversion to the more stable phase was apparently complete 5 days after the melting of the bulk aqueous phase at 0 °C.

(*B*) *Ditridecanoyl-PC.* The DSC thermogram shown in Figure 3 is an example of the type of behavior exhibited by

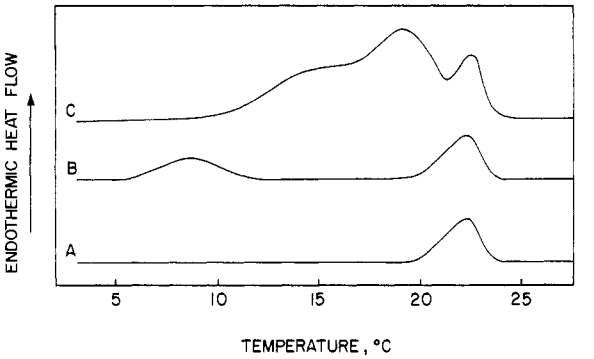

FIGURE 5: Initial DSC heating thermograms of 15:0-PC after various periods of annealing at 0 °C (scan rate = 11.6 °C·h^{-1}). The thermograms shown are drawn on the same y axis and do not include the gel/liquid-crystalline phase transition endotherm. (A) No annealing; (B) 5 days annealing; (C) 2 weeks annealing.

13:0-PC after annealing under the conditions listed in Table I. The DSC thermogram shows evidence of major heating endotherms with maxima occurring at temperatures near 8, 12, 13, and 14 °C, respectively (scan rate 11.6 °C h^{-1}). This is accompanied by a complete abolition of the pretransition which in unannealed samples is observed at temperatures near −1 °C (see Figure 2). The sharp peak observed at temperatures near 13 °C is presumably the result of a $P_{\beta'} \rightarrow L_\alpha$ transition, since its characteristic properties (i.e., T_m, width at half-height, etc.) are the same as those of an unannealed sample (see Figure 1). As with 12:0-PC, there appeared to be a slow growth of the peak at 14 °C at the expense of the other three (data not shown) as the annealing time is extended, and this is accompanied by large increases in the total enthalpy changes (see Table III).

(*C*) *Dimyristoyl-PC.* The DSC thermogram presented in Figure 3 shows that once annealed as described in Table I, the pretransition, which in unannealed samples is normally observed at 14 °C, is replaced by a considerably more energetic transition with a maximum at temperatures near 16 °C. Our unpublished ^{31}P NMR spectroscopic data indicate that the low-temperature phase formed on annealing is an L_c phase. Thus, the observed heating endotherm is the result of a net $L_c \rightarrow P_{\beta'}$ transition. The observed L_c phase is the most stable one that was formed under our conditions, and after being seeded at low temperatures, its formation was apparently complete when incubated as described in Table I.

(*D*) *Dipentadecanoyl-PC.* Figure 3 includes an initial DSC heating thermogram of 15:0-PC after annealing as described in Table I. The thermogram clearly shows the appearance of two additional heating endotherms prior to the onset of the pretransition at temperatures near 22 °C. Figure 5 shows some initial heating endotherms of 15:0-PC after seeding at −10 °C followed by various periods of incubation at 0 °C. It is clear that with incubation at 0 °C there is first the formation of a subgel phase of relatively low enthalpy, which upon heating converts to the $L_{\beta'}$ phase at temperatures near 10 °C. Further incubation at 0 °C results in the formation of other subgel phases which are apparently more stable and upon heating would convert to the $L_{\beta'}$ phase at temperatures near 13 and 17 °C. The conversion to the most stable L_c phase so far observed ($T_m \simeq 17$ °C) is a process which takes up to 6 weeks of incubation at 0 °C.

(*E*) *Long-Chain PCs* (*n* = *16–22*). Figure 3 also shows some initial DSC heating endotherms obtained after samples of these long-chain PCs were incubated at 0–4 °C for extended periods (≃16 months). There is clear evidence that all of the long-chain PCs formed some kind of an L_c phase. For samples

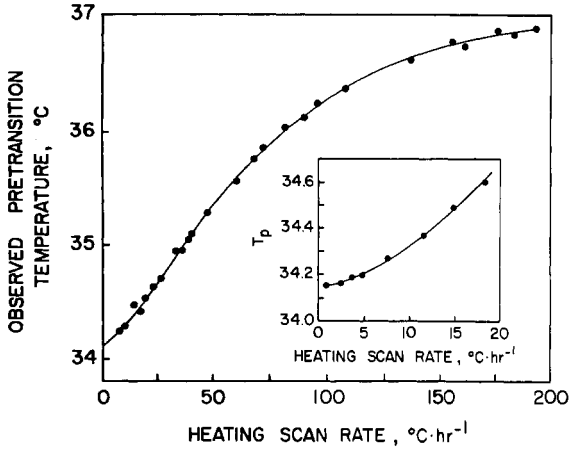

FIGURE 6: Effect of scan rate on the peak heating endothermic transition temperature of the pretransition of dipalmitoylphosphatidylcholine. The inset is a scale expansion of the slow scan rate region of the curve.

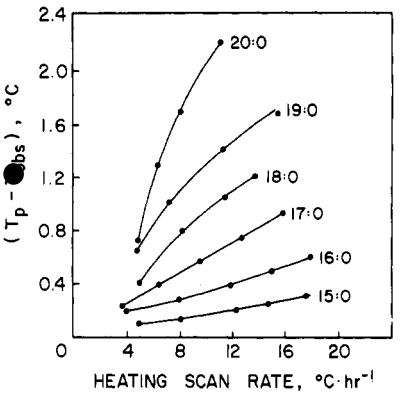

FIGURE 7: Effect of scan rate on the peak heating endothermic transition temperatures of the pretransition of 1,2-di-*n*-acyl-PCs. To facilitate comparison of the data, the curves are all offset on the *y* axis, and only the initial rise phases of the curves are plotted.

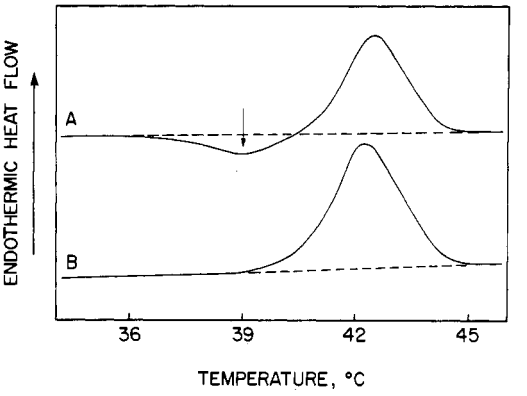

FIGURE 8: Effect of cooling rate on the pretransition heating endotherm of 17:0-PC. The endotherms shown were recorded at 5.2 °C·h^{-1} after (A) fast cooling ($\simeq$10 °C·min^{-1}) and (B) slow cooling ($\simeq$1 °C·h^{-1}).

like 16:0-PC and 17:0-PC, single subtransition endotherms are observed, while all of the others, with the probable exception of 21:0-PC, exhibit two endothermic subtransition like events. When the subtransition behavior of 16:0-PC and 17:0-PC was examined as a function of annealing time at 0–4 °C, it was found that the subtransitions did occur at the temperatures previously reported in the literature. However, with prolonged incubation at 0–4 °C, there is a progressive growth of an apparently more stable phase at the expense of the L_c phase which is initially formed. This is accompanied by an increase in the total enthalpy and in the calorimetrically observable transition temperature. Under our conditions, the conversion to the more stable L_c phases of 16:0-PC and 17:0-PC is complete after 8–12 weeks of incubation at 0–4 °C. Similar processes are probably occurring in all of the long-chain PCs studied albeit on a considerably longer time scale. It is clear from the heating thermograms shown in Figure 3 that all of the longer chain PCs ($n \geq 18$) do form some type of L_c phase, and this is supported by our unpublished ^{31}P NMR spectroscopic data. Under our conditions, the formation of their L_c phases was initiated after a few weeks of incubation at 0–4 °C, and the transformation to the more stable L_c phase was obviously not complete after 16 months. Over the 16-month annealing period used in this study, we found that the extent of conversion to the stable L_c phase decreased with increasing acyl chain length, an observation which is indicative of slower kinetics of transformation with increasing acyl chain length.

Kinetic Aspects of the $L_{\beta'} \rightarrow P_{\beta'}$ Transition. The pretransition of the *n*-acyl-PCs is a slow process, and as a result, temperature scanning techniques tend to observe the process at temperatures higher than its true equilibrium transition temperature [see Silvius (1982) and references cited therein]. Many workers have attempted to obtain estimates of the true equilibrium pretransition temperature (T_p) by measuring T_p as a function of heating rate and extrapolating the apparently linear function to zero scan rate. We have attempted such an approach, and the results of one of these studies are shown in Figure 6. It is clear that T_p does not vary linearly with the heating rate and it is best described by a sigmoidal function. The mean slopes of the initial rise phases of the curves increased with increasing acyl chain length, ranging from values near 0.03 °C·(deg/h)$^{-1}$ for 15:0-PC to values near 0.1 °C·(deg/h)$^{-1}$ for 20:0-PC.

We also investigated the effect of cooling rate on the pretransition so as to enable an evaluation of whether there was adequate preequilibration between the $L_{\beta'}$ and $P_{\beta'}$ phases under our sample preparation conditions. We found that the calorimetric observability of the pretransition was severely compromised by fast cooling rates, especially in the case of the longer chain odd-numbered PCs. At the slowest cooling rates used (18.75 °C·h^{-1}), the pretransition cooling exotherms were easily observable in the case of the shorter chain PCs (n = 13–16) but barely detectable in the case of the longer chain odd-numbered PCs (n = 17, 19 21; see Figure 2). Furthermore, quick cooling of a sample (especially the longer chain odd-numbered PCs) frequently results in the occurrence of an exotherm on the succeeding DSC thermogram at temperatures just below the onset of the pretransition endotherm (see Figure 8). The above observations are indicative of a tendency toward slower rates of interconversion between the $L_{\beta'}$ and $P_{\beta'}$ phases as the acyl chain length increases, and they also suggest that the kinetics of the process may be generally slower with the odd-numbered compounds. Indeed, the observations described above suggest that for the longer chain, odd-numbered PCs, the rates of $L_{\beta'} \leftrightarrow P_{\beta'}$ interconversion are sufficiently slow that quick cooling of the sample would result in some kinetic trapping of the $P_{\beta'}$ phase at temperatures well below the range in which it is thermodynamically stable. A similar exotherm was also observed in the DSC heating thermograms of samples of 22:0-PC that were quench cooled. Thus, for 22:0-PC, the single heating endotherms shown in Figures 1 and 2 must contain some contribution from a kinetically limited process like the pretransition.

Thermodynamic Characterization. The calorimetrically determined peak temperatures assigned to the various thermotropic transitions exhibited by these PCs are listed in Table II, and the measured enthalpy changes associated with those

Table IV: Thermodynamic Properties of Gel/Liquid-Crystalline Phase Transitions of 1,2-Di-*n*-acylphosphatidylcholines

PC	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH_c (kcal·mol^{-1})	ΔH_v (kcal·mol^{-1})	CU
13:0	13.7	~0.1[c]	4.4	5.76×10^3	1309
14:0	23.9	~0.12[c]	5.9	5.15×10^3	872
15:0	34.7	~0.1[c]	6.9	6.63×10^3	961
16:0	41.4	~0.13[c]	7.7	5.33×10^3	691
17:0	49.8	0.16	8.7	4.7×10^3	540
18:0[a]	55.3	0.24	9.8	3.15×10^3	321
19:0[a]	61.8	0.24	10.7	3.27×10^3	306
20:0[a]	66.4	0.32	11.4	2.52×10^3	221
21:0[a]	71.1	0.47	12.2	1.76×10^3	145
22:0[a,b]	74.8	0.53	14.9	1.6×10^3	107

[a] There was some hydrolysis of these samples during the DSC experiment. Consequently, the $\Delta T_{1/2}$ values quoted are overestimates of the true value; the ΔH_c values are underestimates of the true value; the ΔH_v values are underestimates of the true values, and the cooperative unit sizes (CU) are underestimates of the true values. The van't Hoff enthalpies (ΔH_v) and cooperative unit sizes (CU) were calculated as described by Mabrey and Sturtevant (1978). [b] The gel/liquid-crystalline phase transition of this PC is believed to be an $L_{\beta'} \rightarrow L_\alpha$ transition. [c] The transitions were too sharp for the $\Delta T_{1/2}$ to be accurately measured on our Microcal MC-1 calorimeter. The real values are probably smaller than those quoted.

events are listed in Table III. With the exception of the $P_{\beta'} \rightarrow L_\alpha$ transition (gel/liquid-crystalline phase transition), all of the observed transitions were subject to some kinetic limitations, and as a result, the transition temperatures listed in Table II (except those pertaining to the $P_{\beta'} \rightarrow L_\alpha$ transitions) are most probably not true equilibrium temperatures. Furthermore, the slow rate of formation of the L_c phases of many of these compounds, as well as the complexity of the process, inevitably led to some uncertainty as to the nature of the L_c phases that were formed and the extent of their conversion to their thermodynamically stable states. For some of these compounds (e.g., the short-chain PCs), it is possible that the thermodynamically stable L_c phase was completely formed, while this was certainly not the case in the longer chain compounds. Given the above, a strict thermodynamic analysis of the transitions involving transformations of the L_c and $L_{\beta'}$ phases was not feasible, and such derived data are only reported for the $P_{\beta'} \rightarrow L_\alpha$ transitions (see Table IV). The data shown in Table IV indicates that the $P_{\beta'} \rightarrow L_\alpha$ transition is a highly cooperative event as judged by the magnitude of the van't Hoff enthalpies and cooperative unit sizes calculated. The values reported for the longer chain PCs ($n \geq 18$) are probably underestimated, since a combination of their high transition temperatures and the slow scan rates at which their endotherms were recorded resulted in low levels of hydrolysis during the DSC runs. However, there is a clear trend showing a decrease in the size of the cooperative units as the length of the acyl chain increases. This probably indicates that the formation of stable microdomains from which interconversions between the $P_{\beta'}$ and L_α phases are nucleated requires progressively fewer PC molecules as the acyl chain length increases. As shown in Figure 9, the chain length dependence of the $P_{\beta'} \rightarrow L_\alpha$ transition temperatures describes a smooth curve with no evidence of odd/even discontinuities. This is what is expected of a simple chain-melting process comparable to the so-called α-melt of *n*-alkanes, for which the melted phase is nucleated from a loosely packed phase in which the hydrocarbon chains are aligned along the normal to the end-group planes [see Broadhurst (1962)]. The enthalpy of the $P_{\beta'} \rightarrow L_\alpha$ transition increases with increasing acyl chain length, and when plotted as a function of the transition temperature (see Figure 10), the data are best described by a smooth curve and not a linear function as has been suggested by previous

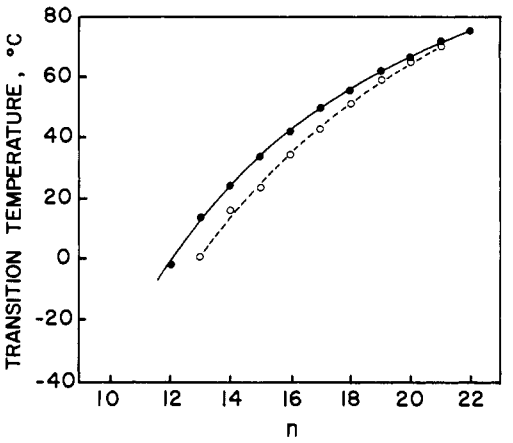

FIGURE 9: Effect of chain length on the peak temperature of the $L_{\beta'} \rightarrow P_{\beta'}$ transition (open circles) and the $P_{\beta'} \rightarrow L_\alpha$ transition (closed circles) of 1,2-di-*n*-acyl-PCs.

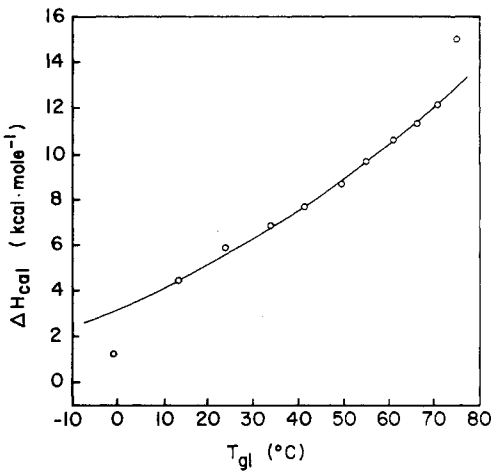

FIGURE 10: Enthalpies of the $P_{\beta'} \rightarrow L_\alpha$ transition plotted as a function of the transition temperature.

studies (Mabrey & Sturtevant, 1976, 1978). It is interesting, however, that the transition enthalpy of 22:0-PC obtained by interpolation on the curve is some 1.5–1.6 kcal·mol^{-1} lower than that observed for the enthalpy of the chain-melting phase transition of that phospholipid. The difference between the observed value and that predicted by the curve is in the range expected of an $L_{\beta'} \rightarrow P_{\beta'}$ transition (i.e., the pretransition).

Discussion

This study of the polymorphic phase behavior of the 1,2-di-*n*-acyl-PCs has shown that all of the PCs studied can form some kind of an L_c phase. This finding is at variance with some of the conclusions of a previously published study (Finegold & Singer, 1986), which suggested that the formation of such phases for PCs with acyl chains of more than 20 carbon atoms is very unlikely. Our study indicates that under appropriate conditions 21:0-PC and 22:0-PC can form L_c phases, albeit very slowly. Our data also suggest that the formation of the L_c phases of *n*-acyl-PCs is a complex process which probably proceeds via a number of metastable intermediates, after nucleation by incubation at suitable low temperatures. This process is also influenced by factors (e.g., sample concentration, handling, purity, and history) in ways which are not fully understood. Thus, the conditions listed in Table I utilized to induce the formation of the L_c phases of these PCs may not be ideal. However, once fully nucleated, there is probably a relatively rapid growth of an initial metastable L_c phase which, upon further incubation at suitable temperatures, slowly transforms into a stable L_c phase via one or more

metastable L_c phase intermediates. This interpretation would account for the multiplicity of "subtransition endotherms" reported here and in other studies (Finegold & Singer, 1984; Silvius et al., 1985). We also expect that, for each of these PCs, there is a relatively narrow temperature window within which the nucleation and perhaps the initial growth of the L_c phases would proceed at a reasonable rate. Thus, for example, the formation of an L_c phase of DPPC could be initiated by incubation at 0–4 °C for 3 days but was not induced by incubation at −8 to −10 °C for 2 weeks. Furthermore, as has been observed with some isoacyl-PCs (Yang et al., 1986), the growth of the initial L_c phases and their eventual transformation to more stable L_c forms often proceed at a faster rate at temperatures higher than those at which they were first nucleated.

Although the slow rates of transformations involving the L_c phases of these lipids were not unexpected [see Chen et al. (1980), Fuldner (1981), Cameron and Mantsch (1982), and Nagle and Wilkinson (1982)], these studies have also shown that the kinetics of all aspects of the formation of the L_c phases become slower as the length of the acyl chain increases. Thus, for the shorter chain PCs (n = 10, 11), apparently stable L_c phases could be formed in 30–45 min, while for their longer chain counterparts (n = 21, 22) the process was not complete in 16 months and may very well take several years. Furthermore, there was some evidence that the kinetics of formation of the L_c phases of the odd-numbered members of this homologous series of PCs are somewhat slower than those of their even-numbered counterparts. This effect was difficult to quantify on account of the extremely slow rate of formation of the L_c phases of many of these compounds. However, a comparison of the calorimetric data recorded for the $L_c \rightarrow L_{\beta'}$ transitions of 21:0-PC and 22:0-PC (see Tables II and III) suggests that inspite of the chain length effects, under identical conditions the formation of the stable L_c phase of 22:0-PC was certainly closer to completion than that of 21:0-PC. Furthermore, we have observed that under identical conditions the formation of the apparently stable L_c phases of 14:0-PC proceeds more quickly than that of 13:0-PC. Interestingly, we have often observed two metastable, L_c phase intermediates in the formation of the apparently stable L_c phases of some of these odd-numbered PCs (n = 13, 15), while for their even-numbered counterparts (n = 12, 14) this process apparently proceeded via one metastable, L_c phase intermediate. Thus, it is possible that the formation of stable L_c phases of the odd- and even-numbered n-acyl-PCs may proceed via different mechanistic pathways. The occurrence of odd/even discontinuities in the kinetics or mechanism of the formation of the L_c phases of these PCs should not be surprising, since it is expected from X-ray diffraction studies [see Ruocco and Shipley (1982)] that in the L_c phase, the hydrocarbon chains of PCs like DPPC are tilted to the bilayer normal. Such odd/even discontinuities are thought to arise naturally from the differences in the possible end-group interactions of crystalline or quasi-crystalline odd- and even-numbered homologues of long-chain paraffinic compounds in which the hydrocarbon chains are tilted to the end-group planes [see Broadhurst (1962)].

The present study also provides new information about the pretransition of the n-acyl-PCs and about the $L_{\beta'}$ and $P_{\beta'}$ phases formed by these compounds. First, we demonstrate for the first time the occurrence of a pretransition in aqueous dispersions of 13:0-PC and 21:0-PC, and we also present evidence that the structural events associated with the $L_{\beta'} \rightarrow P_{\beta'}$ transition probably occur in the case of 22:0-PC even though, in the heating mode, the process is not demonstrably separate from the chain-melting phase transition of that lipid. Second, we also show that the $L_{\beta'} \rightarrow P_{\beta'}$ transition becomes slower as the acyl chains lengthen and provide evidence for odd/even discontinuities in the kinetics of that process (the pretransition). These observations suggest that in either the $L_{\beta'}$ or the $P_{\beta'}$ phase (more likely the $L_{\beta'}$ phase), the hydrocarbon chains of either the odd- or the even-numbered PCs (or both) are inclined to the bilayer normal, a suggestion consistent with previously published X-ray data [see Church et al. (1986)]. In addition, the slow rates of interconversion between the $L_{\beta'}$ and $P_{\beta'}$ phases and the fact that it is possible to kinetically trap some elements of the $P_{\beta'}$ phase at temperatures below the range of their thermodynamic stability (especially in the case of the longer chain odd-numbered homologues) underscore the fact that an accurate determination of the properties of the $L_{\beta'} \rightarrow P_{\beta'}$ transition requires care in sample preparation in order to ensure complete conversion to the L_c phase when the sample is annealed at low temperatures. We have observed that any kinetic trapping of elements of the $P_{\beta'}$ phase within the $L_{\beta'}$ phase severely inhibits the processes by which the L_c phase is formed. Third, these studies indicate that with decreasing acyl chain length, the $P_{\beta'}$ and $L_{\beta'}$ phases of these PCs become increasingly more unstable with respect to one or more of their L_c phases. In this study, we have probably not observed the $L_{\beta'}$ phase of 12:0-PC and the $L_{\beta'}$ or $P_{\beta'}$ phases of either 10:0-PC or 11:0-PC. We suspect that this is largely due to a combination of the unstability of those phases with respect to their respective L_c phases as well as to the relatively rapid rate at which their L_c phases form at the low temperatures that are probably necessary for the formation of their respective $P_{\beta'}$ and $L_{\beta'}$ phases. Fourth, we have confirmed the conclusions of a previously published study (Finegold & Singer, 1986) that the $L_{\beta'}$ and $P_{\beta'}$ phases of 13:0-PC and the $L_{\beta'}$ phase of 14:0-PC and 15:0-PC are metastable at all temperatures at which they have been observed: i.e., their apparent stability at those temperatures is largely due to the fact that nucleation of their L_c phases takes place at temperatures below the freezing point of water. Given the above, we have classified the various PCs used in this study on the basis of the relative stabilities of their L_c, $L_{\beta'}$, and $P_{\beta'}$ phases and the nature of the heating endothermic transitions expected at equilibrium (see Figure 11).

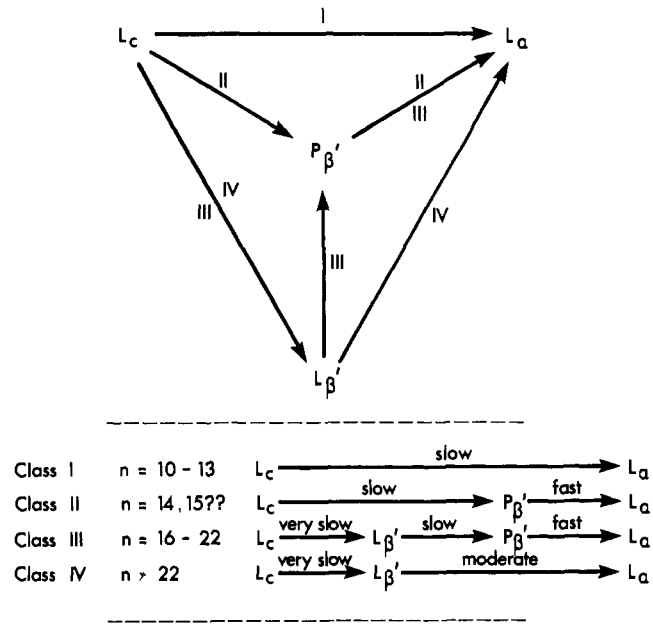

FIGURE 11: Acyl chain length and thermal decomposition of the L_c phases of 1,2-di-n-acyl-PCs.

These results suggest that with an increase in acyl chain length, the change in the relative thermal stabilities of the L_c, $L_{\beta'}$, and $P_{\beta'}$ phases of these PCs is such that the main structural events involved in the conversion of the L_α phase become separated on the temperature scale into the events commonly known as the subtransition, pretransition, and gel/liquid-crystalline phase transition.

Although the *n*-acyl-PCs described here can all form L_c phases, they appear to be less prone to form such structures than are the isoacyl- and ω-cyclohexyl-PCs [see Lewis and McElhaney (1985a,b), Mantsch et al. (1985), Church et al. (1986), and Yang et al. (1986)] or a number of saturated mixed-chain PCs [see Stumpel et al. (1983), Lewis et al. (1984), Serrallach et al. (1984), Tummier et al. (1984), and Hui et al. (1984)]. Obviously, there is a significant kinetic component to this behavior, and this makes it difficult to determine whether there is an underlying thermodynamic trend. We suspect that the physical basis of the above observations, as well as the greater tendency of the shorter chain homologues to form such L_c phases, probably lies in the packing and interactions of the end groups on the hydrocarbon chains. It has become apparent from a number of studies that the packing requirements of the end groups on hydrocarbon chains are major determinants of their "solid-phase" properties and that the accomodation of those end groups often results in the formation of condensed structures with strong tilting of the hydrocarbon chains to the end-group planes [see Malkin (1952), Ishizawa (1971a,b), and Ishizawa et al. (1986a,b)]. Such studies have suggested that the end-group effects are dependent on factors like the size and polarity of the end groups and tend to be moderated as the length of the hydrocarbon chain is increased. For the *n*-acyl-PCs, the relatively small size of the ω-methyl end group may in part account for the observed behavioral differences. Such considerations have not been thoroughly addressed in the literature, and the few attempts made have approached the problem from an empirical perspective [see Davis and Keough (1985) and Mason and Huang (1981)]. Thus, a fuller understanding of the phase behavior of these PCs and other lipids will require a better definition of the contributions of the interchain and end-group interactions to the stability of the PC bilayer as well as the dependence of these and other interactions on the nature of the solvent present.

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