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# Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing Iso-Branched Fatty Acids. 1. Differential Scanning Calorimetric Studies†

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ABSTRACT: The thermotropic phase behavior of aqueous dispersions of phosphatidylcholines containing one of a series of methyl iso-branched fatty acyl chains was studied by differential scanning calorimetry. These compounds exhibit a complex phase behavior on heating which includes two endothermic events, a gel/gel transition, involving a molecular packing rearrangement between two gel-state forms, and a gel/liquid-crystalline phase transition, involving the melting of the hydrocarbon chains. The gel to liquid-crystalline transition is a relatively fast, highly cooperative process which exhibits a lower transition temperature and enthalpy than do the chain-melting transitions of saturated straight-chain phosphatidylcholines of similar acyl chain length. In addition, the gel to liquid-crystalline phase transition temperature is relatively insensitive to the composition of the aqueous phase. In contrast, the gel/gel transition is a slow process of lower cooperativity than the gel/liquid-crystalline phase transition and is sensitive to the composition of the bulk aqueous phase. The gel/gel transitions of the methyl iso-branched phosphatidylcholines have very different thermodynamic properties and depend in a different way on hydrocarbon chain length than do either the "subtransitions" or the "pretransitions" observed with linear saturated phosphatidylcholines. The gel/gel and gel/liquid-crystalline transitions are apparently concomitant for the shorter chain iso-branched phosphatidylcholines but diverge on the temperature scale with increasing chain length, with a pronounced odd/even alternation of the characteristic temperatures of the gel/gel transition. Our observations can be rationalized by assuming that the stable conformations characteristic of these lipids at temperatures below the gel/gel transition temperature and their conformation in the liquid-crystalline state are such that direct interconversions between the two states are improbable and that such interconversions must proceed via an intermediate state or states.

Fatty acids containing a single methyl branch near the methyl terminus of the hydrocarbon chain are abundant and widespread constituents of the membrane lipids of eubacteria. In addition to their occurrence in all species of the genus *Bacillus* which have been examined thus far, methyl iso- and anteiso-branched fatty acids are also found in eight other genera of Gram-positive and in four genera of Gram-negative eubacteria. In those bacterial species in which they occur, methyl iso- and anteiso-branched fatty acids are normally the predominant components of the membrane lipids, accounting for between 65% and 95% of the total esterified fatty acid; even-chain saturated fatty acids, especially palmitic acid,

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account for most of the balance, and monounsaturated fatty acids are often absent entirely or present in relatively small amounts. Like linear saturated fatty acids, methyl iso-branched fatty acids have relatively high melting points and tend to be esterified predominantly at the 1-position of the glycerol backbone of bacterial membrane glycerolipids while, like monounsaturated fatty acids, methyl anteiso-branched fatty acids have relatively low melting points and are located primarily at position 2. Moreover, as the growth temperature is increased, the proportion of iso-branched (and straight-chain saturated) fatty acids found in the membrane lipids usually increases whereas the proportion of anteiso-branched (and monounsaturated) fatty acids invariably decreases. It has been suggested that methyl iso- and anteiso-branched fatty acyl groups perform similar functions in the lipids of bacterial membranes as do linear saturated and monounsaturated hydrocarbon chains, respectively [for a review, see Kaneda (1977)].

In addition to being abundant and widely distributed in the eubacteria, methyl iso- and anteiso-branched fatty acids are also able to support the growth of several fatty acid auxotrophic procaryotic microorganisms in which these fatty acid classes do not naturally occur. For example, the cholesterol-requiring and fatty acid requiring mycoplasma *Mycoplasma mycoides* grows well on single methyl iso- or anteiso-branched fatty acids as well as on several trans-monounsaturated fatty acids, whereas single straight-chain saturated or cis-monounsaturated fatty acids will not support growth (Rodwell & Peterson, 1971). Similarly, the noncholesterol-requiring mycoplasma *Acholeplasma laidlawii* B, when rendered fatty acid auxotrophic, grows well on a variety of methyl iso- and anteiso-branched fatty acids as well as on trans-unsaturated and *trans*-cyclopropane fatty acids, whereas most straight-chain saturated, cis-monounsaturated, and *cis*-cyclopropane fatty acids support little or no growth (Silvius & McElhaney, 1978). Finally, certain methyl iso- and anteiso-branched fatty acids will substitute for cis or trans monounsaturates in supporting the growth of some unsaturated fatty acid auxotrophs of *Escherichia coli* (Silbert et al., 1973). These studies indicate that saturated fatty acids with a methyl branch near the end of the hydrocarbon chain, alone or in combination with other fatty acids classes, possess the proper physical and chemical properties to support normal membrane function in a wide variety of procaryotic microorganisms.

Despite their extensive distribution and experimental usefulness, relatively little is known about the physical properties of phospholipids containing branched-chain fatty acids. Differential thermal analysis (DTA)[1] studies of the thermotropic phase behavior of fully hydrated iso- and anteiso-branched PC's (Silvius & McElhaney, 1979, 1980) and a recent DSC and monolayer study (Kannenberg et al., 1983) have shown that, when compared with a saturated unbranched PC of similar carbon number, the presence of the methyl branch significantly decreases the gel/liquid-crystalline phase transition temperature, reduces the enthalpy change associated with chain melting slightly, and increases the molecular areas occupied by the molecules in the gel state, particularly if that methyl branch is present in the anteiso position. However, much remains to be done to fully characterize the thermotropic phase behavior of PC's containing methyl-branched fatty acyl chains and to elucidate the molecular basis of this behavior. We report here the results of a DSC study of the thermotropic phase behavior of a series of methyl iso-branched PC's. In the following paper (Mantsch et al., 1985), the results of $^{31}P$ NMR and Fourier transform infrared spectroscopic studies of the molecular basis of the thermotropic phase behavior observed by DSC are presented. The results of similar studies of aqueous dispersions of a series of methyl anteiso-branched PC's will be published shortly.

Table I: Precursors to the Synthesis of Iso-Branched Fatty Acids

| fatty acid[a] | carboxyl fragment | alkyl terminal fragment |
|---|---|---|
| $C_{12i}$ | adipic acid monomethyl ester[b] | 1-bromo-4-methylpentane |
| $C_{13i}$ | azelaic acid monomethyl ester[b] | 1-bromo-2-methylpropane |
| $C_{14i}$ | 1,10-decanedioic acid | 1-bromo-2-methylpropane |
| $C_{15i}$ | 1,10-decanedioic acid | 1-bromo-3-methylbutane |
| $C_{16i}$ | 1,10-decanedioic acid | 1-bromo-4-methylpentane |
| $C_{17i}$ | 1,12-dodecanedioic acid | 1-bromo-3-methylbutane |
| $C_{18i}$ | 1,12-dodecanedioic acid | 1-bromo-4-methylpentane |
| $C_{19i}$ | 1,13-tridecanedioic acid | 1-bromo-4-methylpentane |
| $C_{20i}$ | 1,14-tetradecanedioic acid | 1-bromo-4-methylpentane |
| $C_{21i}$ | 1,14-tetradecanedioic acid | 1-bromo-5-methylhexane |
| $C_{22i}$ | 1,14-tetradecanedioic acid | 1-bromo-6-methylheptane |

[a] Fatty acids are denoted by their carbon number ($C_n$) and the subscript "i" to denote iso branching. [b] These monomethyl esters were obtained from Aldrich Chemical Co.

## Materials and Methods

L-$\alpha$-Glycerophosphocholine/cadmium chloride complex was obtained from Sigma and dried in vacuo before use. 4-Pyrrolidinopyridine was synthesized (Vorbruggen, 1972) or purchased from Aldrich and recrystallized from pentane before use. Ethylene oxide and DCCD were obtained from Eastman Kodak Co. All other commercially available reagents were obtained from Aldrich and where necessary were purified and dried by methods appropriate for the given chemical reaction (Perrin et al., 1966). 1-Bromo-2-methylpropane and 1-bromo-3-methylbutane were used to synthesize 1-bromo-4-methylpentane annd 1-bromo-5-methylhexane, respectively, by reacting ethylene oxide with the respective Grignard reagent followed by bromination of the alcohol formed (Vogel, 1957). 1-Bromo-6-methylheptane was synthesized by the bromination of the alcohol formed by the hydroboration/oxidation of 6-methyl-1-heptene (Brown & Rao, 1959). The latter was synthesized by the reaction of allyl bromide with Grignard reagent from 1-bromo-3-methylbutane (Vogel, 1957). Silicic acid (Biosil-A 200–400 mesh) was obtained from Bio-Rad Laboratories, and silica gel G was obtained from Merck. Both chromatographic adsorbents were washed with chloroform and methanol and then reactivated before use. All solvents were distilled before use and where necessary were purified and dried by methods appropriate for the given reactions (Perrin et al., 1966).

*Fatty Acid Synthesis.* The fatty acids used in this study were synthesized by the procedure outlined in Figure 1. A dicarboxylic acid was used to generate the carboxyl end, and a branched chain alkyl bromide was used to generate the alkyl terminal end. The fragments used for the individual syntheses are listed in Table I. The details are as follows:

The dicarboxylic acid (I) was esterified by refluxing a 5% solution of the acid in acidic methanol (5% $H_2SO_4$) for 12 h. The mixture was diluted with water and the diester II extracted with chloroform and purified by distillation under reduced

[1] Abbreviations: PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; DTA, differential thermal analysis; DSC, differential scanning calorimetry; DCCD, dicyclohexylcarbodiimide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; $T_{gg}$, characteristic temperature of the gel/gel phase transition; $T_{gl}$, characteristic temperature of the gel/liquid-crystalline phase transition; $T_m$, characteristic temperature of the main heating endotherm; $T_f$, characteristic temperature of the main cooling exotherm; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

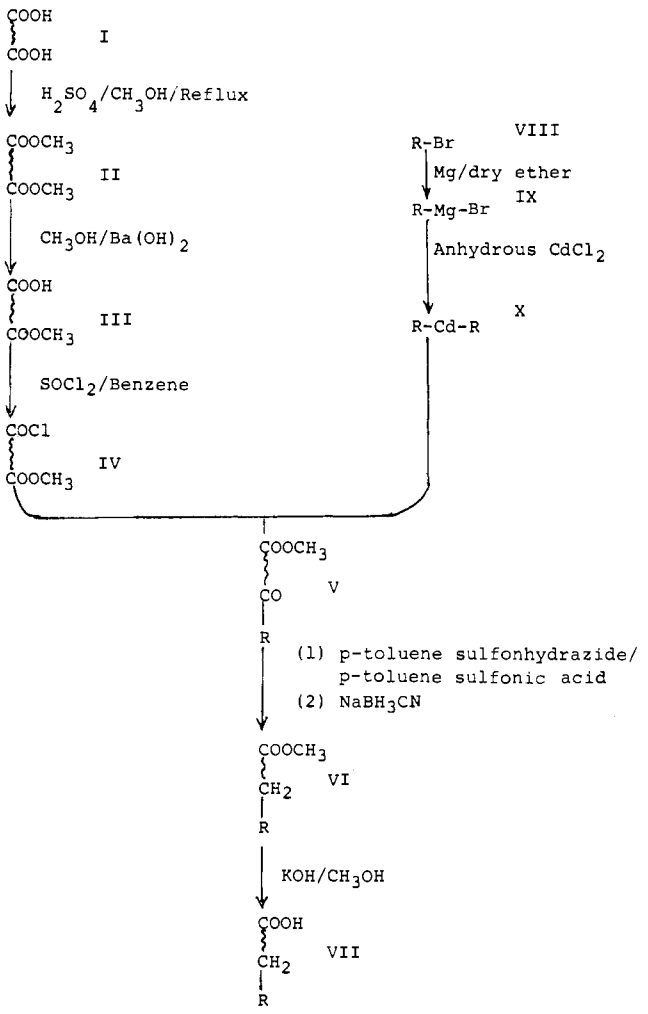


FIGURE 1: Outline of the scheme used for the synthesis of the methyl iso-branched fatty acids. R = branched-chain carbon skeleton.

pressure (≤10 carbons) or recrystallization from methanol (≥12 carbons). The diester II (≥10 carbons) was converted into the monomethyl ester (III) by the method of Durham et al. (1963) and purified by recrystallization form hexane. When the longer chain monomethyl esters (≥13 carbons) were synthesized, the reaction was done at 50 °C because of the low solubility of those diesters in methanol. The acyl chloride (IV) was formed by the slow addition of a solution of III in benzene to a 2-fold excess of thionyl chloride refluxing in benzene. The mixture was refluxed for 30 min, and the solvent and excess thionyl chloride were removed by evaporation under reduced pressure. The acyl chloride was used immediately.

The alkyl bromide (VIII) (2.5 mol per mole of acyl chloride) was converted into the Grignard reagent by standard procedures, and the dry etherate was filtered anhydrously through glass wool into a three-necked flask equipped for mechanical stirring in a dry nitrogen atmosphere under reflux. The etherate was cooled to 0 °C and anhydrous cadmium chloride (0.55 mol per mole of alkyl bromide) added with vigorous mechanical stirring. The mixture was refluxed for 30 min, after which time the formation of the adduct (X) was complete. The apparatus was then set up for distillation under anhydrous conditions and the solvent distilled off with the simultaneous addition of dry benzene and mechanical stirring until the distillation temperature reached 65 °C. The apparatus was then reset for refluxing under anhydrous conditions and cooled to 0 °C. The acyl chloride (IV) was added in dry benzene and the mixture refluxed at 65 °C for 0.5 h with vigorous mechanical stirring. The reaction mixture was then cooled to 0 °C and quenched with methanol. The resulting mixture was diluted with water, acidified to dissolve any precipitate, and extracted with hexane. The hexane phase was washed twice with saturated sodium chloride, dried over anhydrous sodium sulfate, and concentrated by evaporation under reduced pressure. Analysis of the residue by gas-liquid chromatography showed that the conversion of the acyl chloride to the keto ester (V) ranged from 70% to 95% and it was then further purified by silicic acid column chromatography. The keto ester was deoxygenated (Hutchins et al., 1973) and the ester (VI) also purified by silicic acid column chromatography. The fatty acid (VII) was isolated after deesterification and crystallization (Silvius & McElhaney, 1979).

*Synthesis and Purification of Phosphatidylcholines.* The PC's were synthesized by the method of Patel et al. (1979). The crude reaction product from 4 mmol of starting fatty acid was diluted to 35 $cm^3$ with chloroform and applied directly to a 15-g column of silicic acid packed in chloroform. The column was washed with 20 column volumes (900 $cm^3$) of 5% methanol in chloroform and 5 column volumes of 10% methanol in chloroform and developed with 50% methanol in chloroform (v/v). One-column volume fractions were collected and analyzed by TLC (Silvius & McElhaney, 1979). The fractions containing PC (pure by TLC) were pooled, and the solvent was evaporated under reduced pressure. The residue was dissolved in 10 $cm^3$ of methanol and extracted by the solvent partition sequence described by Bligh & Dyer (1959) using 0.1 M HCCOH/HCOONa, pH 3.7, as the aqueous component. The chloroform phase was dried by filtration through chloroform-wetted filter paper and applied to a 10-g column of silicic acid packed in chloroform. The column was washed and developed as described above, the PC fractions (TLC pure) were pooled, and the solvent was removed in vacuo. The residue was dried by azeotropic evaporation with benzene, dissolved in benzene, frozen, and lyophilized. The samples were stored desiccated as a white fluffy powder at −20 °C.

*Differential Scanning Calorimetry.* A sample of the lyophilized PC was hydrated by vortexing for 3–4 min in distilled water at a temperature 10 °C above that of its main phase transition. Aliquots (1 $cm^3$) of the hydrated sample were heated in a Microcal MC-1 high-sensitivity differential scanning calorimeter at rates suitable for the resolution of the complex thermotropic phase behavior of the PC's (5–25 °C $h^{-1}$). Low-sensitivity DSC runs were performed in a Perkin-Elmer DSC-2C differential scanning calorimeter equipped with a thermal analysis data station. The samples were quantitated by gas-liquid chromatography of the methyl esters derived by transesterification using a suitable PC as an internal standard. The esters were analyzed on a diethylene glycol succinate column in a Hewlett Packard 5700A gas chromatograph equipped with a Hewlett Packard 3390 integrator.

## RESULTS

*Chemical Syntheses and Purification of Phosphatidylcholines.* The fatty acids were synthesized in yields of 30–50% based on the monomethyl ester III used. The procedure described here is preferred to that previously used in this laboratory (Silvius & McElhaney, 1979), since the reactions are simpler to execute and, in general, are cleaner with respect to side reactions. In addition, given pure starting materials, the probable side reactions are not expected to produce other monobasic fatty acids as impurities. This is important, since the thermotropic behavior of the respective diacyl-PC's is

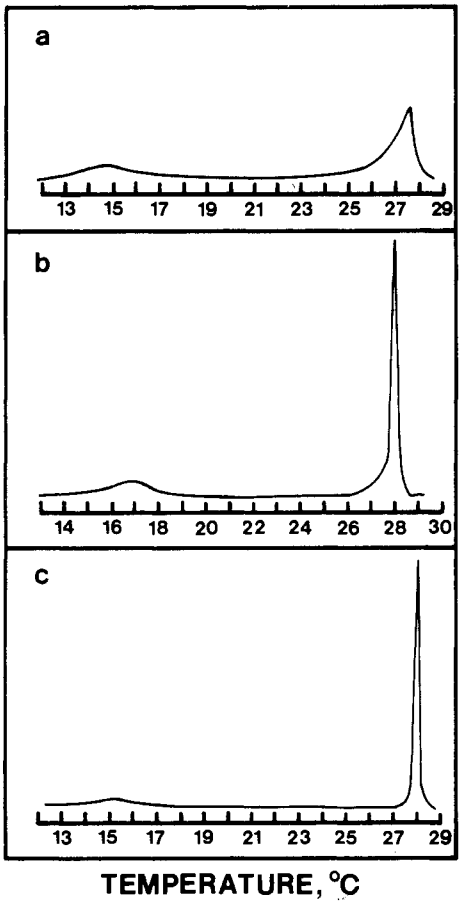


FIGURE 2: High-sensitivity DSC heating thermograms of nominally pure samples of 17i PC. The samples were purified according to Patel et al. (1979) (a) or Silvius & McElhaney (1979) (b) or as described under Materials and Methods (c).

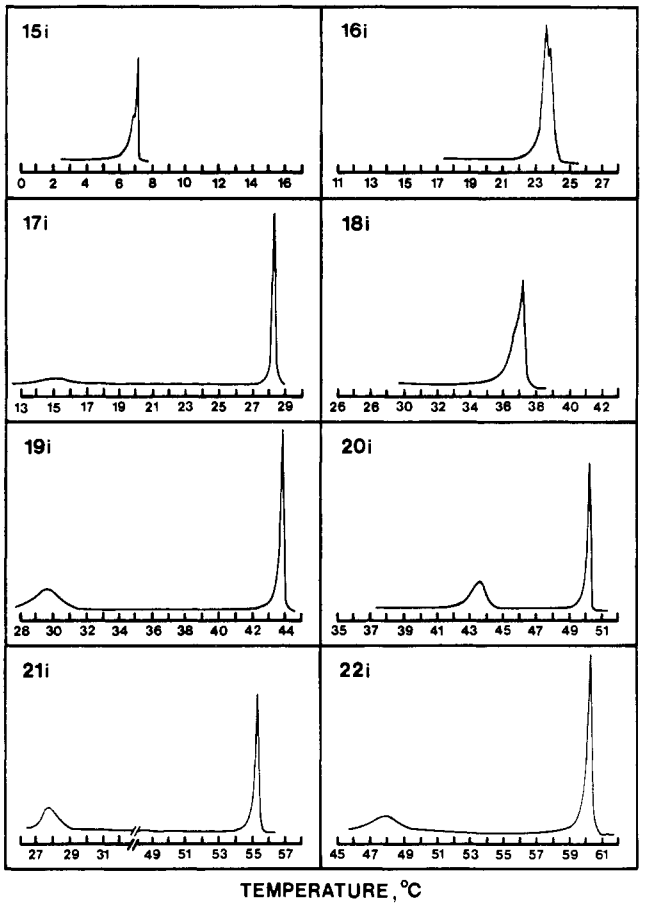


FIGURE 3: High-sensitivity DSC heating thermograms of 1,2-diisoacyl-PC's of $C_{ni} \geq 15$. The thermograms were obtained at the following heating rates: 15i $\simeq$ 5.6 °C $h^{-1}$; 16i $\simeq$ 5 °C $h^{-1}$; 17i $\simeq$ 22 °C $h^{-1}$; 18i $\simeq$ 5.3 °C $h^{-1}$; 19i $\simeq$ 21 °C $h^{-1}$; 20i $\simeq$ 14 °C $h^{-1}$; 21i $\simeq$ 18 °C $h^{-1}$; 22i $\simeq$ 21 °C $h^{-1}$.

extremely sensitive to the presence of impurities and acyl chain inhomogeneity. Our analyses showed that the fatty acids were acyl chain homogeneous (>99.9%) though they did contain some traces of tetramethylene sulfone as an impurity (<0.1%).

The acylation of the L-$\alpha$-glycerophosphocholine proceeded in yields comparable to those described by Patel et al. (1979). The purification of these isoacyl-PC's was particularly important since their thermotropic behavior is extremely sensitive to the presence of impurities. Although it was relatively easy to prepare samples which were judged to be TLC pure in a variety of solvent systems, the thermotropic behavior of many of those samples suggested that there was some systematic trace contamination. Figure 2 shows the thermotropic behavior of the $C_{17i}$ [fatty acids are denoted by the number of carbons (e.g., 17) with i denoting iso branching] species which were purified as per Patel et al. (1979) (a), Silvius & McElhaney (1979) (b), or the procedure described above (c), all of which were judged to be chromatographically pure (TLC and HPLC). It is clear that the sample purified as described here is characterized by a greater cooperativity of the main transition near 28 °C. We have since correlated the broadening of the main transition of this species and an increase in the enthalpy of its lower endotherm near 15 °C with the inclusion of low levels of impurities. Given this, and the fact that the cooperativity of the main transition is generally used as an index of purity (Sturtevant, 1982), we are of the opinion that the purification procedure described here is superior to the others with regard to the purity of the final sample. However, given the high sensitivity of these compounds to impurities, it is apparent that the preparation of highly purified samples requires great care and a considerable sacrifice in the yield of the final product. We generally found that the procedure as described is sufficient for the production of very pure samples. However, we routinely repeated the formic acid extraction/column chromatography phase of the described procedure until there was no further increase in the cooperativity of the main transition. Such repetition has rarely been necessary more than once.

*Thermotropic Phase Behavior.* The DSC heating scans of aqueous dispersions of each of the eight methyl iso-branched PC's exhibiting endotherms at temperatures above 0 °C, and which are thus experimentally accessible to study in the high-sensitivity calorimeter, are presented in Figure 3. It is clear that they all exhibit a complex behavior in the sense that in each case two thermal events can be detected by DSC. These events overlap for the $C_{15i}$ and for the $C_{16i}$ and $C_{18i}$ PC's but are completely resolved for the longer chain members of both the odd- and even-chain series. The thermotropic phase behavior of aqueous dispersions of the $C_{12i}$, $C_{13i}$, and $C_{14i}$ PC's was studied in the low-sensitivity calorimeter, and for each of these compounds, a single heating endotherm was recorded.

The methyl iso-branched PC's for which both thermal events are resolved are characterized by a higher temperature transition which is highly cooperative and relatively energetic and exhibits little or no heating or cooling hysteresis under the experimental conditions employed. Subsequent infrared spectroscopic studies have demonstrated that this higher temperature transition is due to a cooperative melting of the hydrocarbon chains [see the following paper (Mantsch et al.,

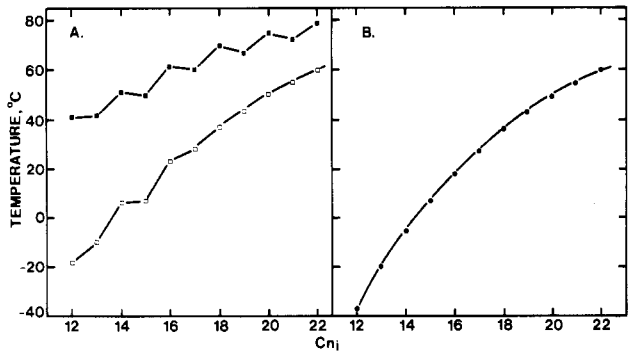


FIGURE 4: (A) Chain-length dependence of the fatty acid melting points (■) and the characteristic temperatures of the main heating endotherms (□) of the 1,2-diisoacyl-PC's. (B) Chain-length dependence of the characteristic temperatures of the cooling exotherms of the 1,2-diisoacyl-PC's.

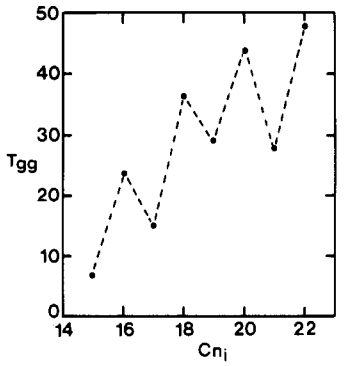


FIGURE 5: Chain-length dependence of the gel/gel transition temperatures of the isoacyl-PC's.

1985)]. The higher temperature thermal event will thus be referred to as the gel/liquid-crystalline phase transition. In contrast, the lower temperature transition is much less cooperative, is generally less energetic, and exhibits a moderate heating and a marked cooling hysteresis, particularly in the case of the two longer chain, odd carbon-number PC's. In fact, the low-temperature transitions of the $C_{19i}$ and $C_{21i}$ PC's are fully manifest only after annealing at 0 °C for 6 days, and these transitions show evidence of kinetic distortion even at quite low heating rates. The degree of separation between the lower and upper temperature transitions increases markedly with increasing chain length. However, separation of these two events occurs at a shorter chain length and is more pronounced in the odd-chain than in the even-chain members of the series. $^{31}P$ NMR and infrared spectroscopic studies [see Mantsch et al. (1985)] have established that the lower temperature transition is due to a gel-state packing rearrangement, and thus this thermal event will be described as the gel/gel phase transition.

The melting temperatures of the methyl iso-branched free fatty acids and the reversal temperatures of the main heating endotherms of the corresponding PC's are plotted as a function of acyl chain number in Figure 4A. The shorter chain PC's appear to show the odd/even alternation characteristic of the melting points of the free fatty acids. However, it is clear that such alternation is not characteristic of the longer chain PC's ($C_{ni} \geq 17$), since the chain-melting transition temperatures of these samples all fall on a smooth curve. Cooling exotherms were recorded by the low-sensitivity Perkin-Elmer DSC-2C calorimeter at the slowest scan rate feasible with this instrument (18.75 °C/h), and the characteristic temperatures were plotted as a function of acyl chain number in Figure 4B. There it was found that *all* the characteristic transition temperatures fall on a smooth curve with no evidence of odd/even alternation. The presence of alternation in the chain-melting transition temperatures on heating and its absence on cooling were suggested by a previous DTA study (Silvius & McElhaney, 1979), which also showed that the heating behavior and cooling behavior of some of these PC's were not fully reversible. A close inspection of Figure 4A,B shows that such is true for four members of the series ($C_{ni}$ = 12, 13, 14, and 16) and that for all other members the heating endotherms and cooling exotherms characteristic of the gel/liquid-crystalline phase transition occur at the same temperature.

A study of the heating and cooling behavior of these isoacyl-PC's suggested that these compounds could be classified into two groups. The first includes the members ($C_{15i}$ and $C_{ni} \geq 17$) for which the midpoint temperatures of the gel/liquid-crystalline phase transition ($T_{gl}$) is demonstrably higher than that of the gel/gel packing transition ($T_{gg}$). For these compounds, $T_{gl}$ was the same in both the heating and cooling modes, and when present, the gel/gel transition was difficult to observe in the cooling mode on account of hysteresis and broadening. The second group ($C_{16i}$ and $C_{ni} \leq 14$) includes those compounds for which $T_{gl}$ is not demonstrably higher than $T_{gg}$. For such compounds, $T_m$, the midpoint temperature of the main heating endotherm, was always greater than $T_f$, the midpoint temperature of the main cooling exotherm. The heating/cooling behavior was carefully studied with the $C_{14i}$ species ($T_m$ = 7.6 °C, $T_f$ = −5.2 °C) to determine whether our observations could be attributable to experimentally induced hysteresis. We found that no exotherm was observed upon cooling an aqueous dispersion of the $C_{14i}$ species from room temperature to 0 °C. Upon reheating the sample from 0 °C, no heating endotherm was observed even if the sample was annealed at 0 °C for 36 h. If the sample were cooled to −20 °C from room temperature, a cooling exotherm was observed at −5.2 °C. Upon reheating to 0 °C, there was no heating endotherm, and a cooling exotherm was not observed upon subsequent recooling from 0 °C even after extended annealing times. Thus, the heating endotherm at 7.6 °C was observed only if the sample was previously cooled to temperatures below −5.2 °C, and the cooling exotherm at −5.2 °C was observed only if the sample was previously heated to temperatures above 7.6 °C. Given these observations, we believe that the observed discrepancy between $T_m$ an $T_f$ is an intrinsic property of these shorter chain PC's and not an experimental artifact attributable to hysteresis.

The gel/gel transition temperatures ($T_{gg}$) resolved by high-sensitivity DSC are plotted as a function of acyl chain number in Figure 5. It is clear that these temperatures are not a continuous function of acyl chain number since there is a pronounced odd/even alternation which does not tend to converge in the range of chain lengths studied. A close inspection of Figure 5 shows that the gel/gel transition temperature recorded for the 21i PC is lower than that recorded for the 19i PC. However, the gel/gel transitions of these two compounds were subject to a pronounced cooling hysteresis and were only fully manifest after prolonged annealing at 0 °C. X-ray diffraction and dilatometric characterization of the subtransition of DPPC (Fuldner, 1981; Nagle & Wilkinson, 1982) have shown that DSC does not always resolve the "true" phase transition temperature of events which approach equilibrium very slowly. Thus, we suspect that the gel/gel transition temperatures recorded for the 19i and 21i PC's may be overestimated, and consequently, they are regarded as upper limit temperatures. The true equilibrium temperatures may not be accessible by DSC.

Table II: Thermodynamic Parameters Characteristic of the Thermotropic Phase Behavior of Fully Hydrated 1,2-Diisoacylphosphatidylcholines

| PC | gel/gel phase transition | | | gel/liquid-crystalline phase transition | | | $\Delta H_{total}$ (kcal/mol)[c] |
|---|---|---|---|---|---|---|---|
| | $T_{gg}$ (°C) | $\Delta T_{1/2}$ (°C)[b] | $\Delta H$ (kcal/mol)[b] | $T_{gl}$ (°C) | $\Delta T_{1/2}$ (°C)[b] | $\Delta H$ (kcal/mol)[b] | |
| 12i[a] | −18.8 | | | −40 | | | 7.8 |
| 13i[a] | −9.5 | | | −19.5 | | | 13.1 |
| 14i[a] | 7.6 | | | −5.2 | | | 11.6 |
| 15i | 6.7 | | ≃3[f] | 7.0 | | ≃3[f] | 5.9 |
| 16i | 23.4 | | ≃6[f] | 19.5 | | ≃6[f] | 11.8 |
| 17i | 15.1 | ≃2 | 2.2 | 28.4 | 0.16 | 8.1 | |
| 18i | 36.5 | | 8[f] | 37.2 | | 10[f] | 17.5 |
| 19i | 29.4 | ≃2[d] | 6.4 | 43.7 | 0.16 | 11.2 | |
| 20i | 43.7 | 1.0 | 9.4 | 50.3[e] | 0.16[e] | ≃13[e] | |
| 21i | 27.8 | ≃2[d] | 3.6 | 55.3[e] | 0.16[e] | ≃14[e] | |
| 22i | 47.9 | 2.1 | 10.0 | 60.4[e] | ~0.20[e] | ≃16[e] | |

[a] These were analyzed by low-sensitivity DSC. $T_{gg}$ and $T_{gl}$ for these and the $C_{16i}$ species were assigned from heating endotherms and cooling exotherms (see text). [b] $\Delta T_{1/2}$ = width at half-height of heating endotherm. These values and the associated enthalpy changes ($\Delta H$) are only reported when there is a base-line separation of the two transitions. [c] This value is reported when the two transitions are not completely resolved. It is expected to be the sum of the enthalpies of both transitions. [d] These gel/gel transitions were only observed after prolonged incubation at low temperatures. The $T_{gg}$ values may be overestimated on account of kinetic artifacts. [e] The high temperatures and slow scan rates necessary for studying the chain-melting transitions of the longer chain PC's resulted in a small amount of hydrolysis (<1%) during each DSC run, with a resultant progressive small decrease in $T_m$ and $\Delta H$ and an increase in $\Delta T_{1/2}$. The reported values were corrected by extrapolation for the effects of this hydrolysis. [f] These values are estimates of the range of enthalpies for the given transitions for those PC's in which there is incomplete resolution. The rationale for these estimates is described in the text.

*Effects of Ethylene Glycol.* While studying the thermotropic behavior of the $C_{14i}$ species suspended in 50% aqueous ethylene glycol (v/v), we found some discrepancy between the midpoint temperatures of the heating endotherms in water and in aqueous ethylene glycol ($T_m$ = 4.5 °C in aqueous ethylene glycol). There was no discrepancy between the midpoint temperatures of the cooling exotherms. Since aqueous ethylene glycol is often used as a suspension medium for samples which have phase transitions near or below the freezing point of water, the effect of ethylene glycol on the phase behavior of these PC's was investigated. We found that the effect of ethylene glycol on the thermotropic behavior of these compounds was dependent upon whether $T_{gl}$ was demonstrably higher than $T_{gg}$. For those compounds with $T_{gl}$ higher than $T_{gg}$, it was found that the properties of the gel/liquid-crystalline transition were unaffected while the heating endotherms of the gel/gel transitions were observed at temperatures lower than those seen in water. The difference between the temperatures varied with scan rate, but never exceeded 3 °C. The cooling exotherms were easier to measure in 50% aqueous ethylene glycol as they were sharper than in water. The enthalpies of the gel/gel transition were not affected in the heating mode, but lower values were recorded in the cooling mode, in both solvents, on account of hysteresis and broadening. In cases where $T_{gl}$ was not demonstrably higher than $T_{gg}$, the effects of ethylene glycol on the heating endotherms were similar to the effects described for the heating endotherms of the gel/gel transitions. The presence of 50% aqueous ethylene glycol had no apparent effects on the cooling exotherms of those compounds.

*Thermodynamic Characterization.* The transition temperatures and the associated enthalpy changes of the heating endotherms of the series of isoacyl-PC's studied are listed in Table II along with the widths at half-height ($\Delta T_{1/2}$) of those high-sensitivity DSC thermograms for which there is complete separation of the gel/gel transition and the gel/liquid-crystalline phase transition on the temperature scale. Although the picture is clouded somewhat by the obvious overlap of these two processes for some of the PC's, it is clear that, when completely resolved, the gel/liquid-crystalline phase transition is a highly cooperative process as evidenced by the small $\Delta T_{1/2}$ values observed. In fact, the sharpness of the chain-melting transition of these methyl iso-branched PC's is quite comparable to that reported for linear saturated PC's of comparable purity [see McElhaney (1982)]. The sharpness of the isoacyl-PC gel/gel transitions is much lower, with $\Delta T_{1/2}$ values for most compounds being about 2 °C. These $\Delta T_{1/2}$ values are similar to those reported for the sub- and pretransitions of DPPC (Chen et al., 1980).

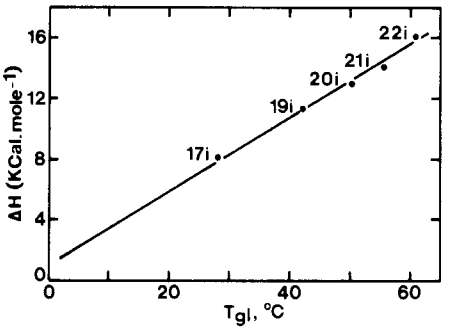


FIGURE 6: Relationship between the enthalpy of the gel/liquid-crystalline phase transition and the gel/liquid-crystalline phase transition temperature of the isoacyl-PC's.

The enthalpies of the resolved gel/liquid-crystalline phase transitions are generally similar but significantly lower than those usually reported for linear saturated PC's of comparable chain length [see Silvius (1982)]. These transition enthalpies increase with acyl chain length and can probably be described by a linear function of the gel/liquid-crystalline phase transition temperature ($T_{gl}$), as shown in Figure 6. Such a relationship has been suggested for the saturated straight-chain PC's (Mabrey & Sturtevant, 1978). Although there is some doubt about the absolute validity of such a relationship [see McElhaney (1982)], the linear function described in Figure 6 may be useful in estimating the enthalpies of the gel/liquid-crystalline and the gel/gel phase transitions of those PC's for which the two processes are not completely resolved. These estimates are listed in Table II. An examination of these estimates along with the other measured enthalpies listed in Table II shows that the range of the enthalpies of the gel/gel transition of the even-numbered members (6–10 kcal/mol) is significantly higher than that of the odd-numbered members of the series (2–6 kcal/mol). This can be related to the pronounced odd/even alternation in the gel/gel transition temperatures shown in Figure 5. It is also clear from Figure 6 that the linear function described cannot be extrapolated to include the shorter chain PC's ($C_{ni}$ = 12–14) and return ac-

ceptable values for the enthalpies of the gel/liquid-crystalline phase transitions of those compounds. Furthermore, the calorimetrically determined enthalpies of the heating endotherms of those short-chain compounds (see Table II) are considerably larger than those determined for the gel/liquid-crystalline phase transitions of the saturated straight-chain compounds of comparable acyl chain number [see Silvius (1982)]. Thus, it is unlikely that the gel/liquid-crystalline phase transition is the only component of the single endotherm characteristic of the thermotropic phase behavior of the shorter chain isoacyl-PC's. Given this and the observed tendency toward increased overlap of the gel/gel and the gel/liquid-crystalline phase transitions with decreasing acyl chain length, it seems likely that the single endotherm observed for these short-chain PC's is a complex process involving both the gel/gel and the gel/liquid-crystalline phase transitions. Furthermore, the contribution of the gel/liquid-crystalline phase transitions of these short-chain PC's to the observed enthalpy changes is expected to be small ($\leq$3 kcal/mol) and as such should not play a major role in the stabilization of the gel-state conformations of these shorter chain PC's. Given the above, it seems that the probable enthalpies of the gel/gel transitions of these shorter chain PC's are discontinuous with, and considerably larger than, those which may be extrapolated from the trends set by the longer chain members ($C_{ni} \geq 15$).

## Discussion

The thermodynamic properties of the gel/gel transition exhibited by the longer chain methyl iso-branched PC's differ considerably from those of either the subtransition or the pretransition characteristic of linear saturated chain PC's of similar hydrocarbon chain length. The subtransition temperature has been reported to exhibit only a small acyl chain length dependence, with the result that the subtransition and main transition show a marked divergence with increases in PC hydrocarbon chain length. In contrast, the pretransition temperature is strongly chain-length dependent, such that the pretransition and chain-melting transition tend to converge as the fatty acyl chains become longer. The methyl iso-branched PC gel/gel transition temperature shows an "intermediate" behavior, increasing moderately with hydrocarbon chain length but not as rapidly as the gel/liquid-crystalline phase transition temperature, so that some divergence between these transition temperatures is observed. In addition, the *n*-saturated PC subtransition and pretransition temperatures exhibit only weak odd/even alternation which disappears with increasing chain length. In contrast, the gel/gel transition temperatures show a marked odd/even alternation which becomes progressively larger with increasing acyl chain length. The enthalpy of the subtransition of DPPC has been reported as 3.0–3.5 kcal/mol, with enthalpy values apparently decreasing with increases in acyl chain length, while the pretransition enthalpy is about 0.9–1.5 kcal/mol and is chain-length invariant. The enthalpies of the gel/gel transition of the isoacyl-PC's are variable but generally are much larger than either the subtransition or the pretransition enthalpies, particularly in the case of the even-chain compounds, and generally increase with increases in hydrocarbon chain length. Finally, the cooperativity of the subtransition of linear saturated chain PC's has been reported to be much lower than that of the pretransition, which exhibits a cooperativity somewhat less than but similar to that of the gel/liquid-crystalline phase transition (Chen et al., 1980). The cooperativity of the gel/gel transition of the methyl iso-branched compounds is much lower than that of the chain-melting transition but is near (even-chain PC's) or slightly greater than (odd-chain PC's) that of the subtransition of the linear saturated chain PC's. These results clearly indicate that the nature of the gel-state transition exhibited by methyl iso-branched PC's is quite different from either of the gel-state transitions exhibited by their linear saturated analogues.

The observed thermotropic behavior of the PC's studied seems to be dependent on whether the gel/gel transition can be resolved from the gel/liquid-crystalline transition on the temperature scale. The separation of the two events increases with increasing chain length though not as a continuous smooth function of chain length. A logical extrapolation of the tendency toward increased overlap of the two events with decreasing chain length is the suggestion that, for the shorter chain compounds, the temperature of the gel/gel phase transition becomes higher than that of the chain-melting transition. For such a system, an inversion of the two events on the temperature scale is not feasible since, at temperatures between $T_{gl}$ and $T_{gg}$, it would require an improbable conformation in which the hydrocarbon chains are melted while still retaining the tightly packed conformation characteristic of the system at temperatues below $T_{gg}$. Thus, it is more likely that for such a system its thermotropic behavior would be a complex phenomenon involving the nearly concomitant occurrence of both physical processes. This could provide a rationalization for our observations if it is further postulated that the stable conformations characteristic of both the liquid-crystalline state and the low-temperature gel state are such that direct interconversion between the two states is improbable and that interconversions between the two states must proceed via an intermediate state or states. The type of intermediate envisaged here would be a loosely packed gel state with extended acyl chains. In the case of the longer chain compounds where $T_{gg}$ is lower than $T_{gl}$, the above postulates would suggest that the intermediate necessary for the interconversion between the two states is stable at temperatures between $T_{gg}$ and $T_{gl}$. Implicit in the above is the assignment of $T_{gg}$ to the temperature favoring interconversion between the stable low-temperature state and the intermediate state, and $T_{gl}$ to the temperature favoring interconversion between the intermediate state and the liquid-crystalline state. In the case of the shorter chain compounds for which $T_{gg}$ is postulated to be higher than $T_{gl}$, the strict requirement for the formation of the intermediate as a necessity for interconversions between the two stable states would result in a difference in behavior upon heating and cooling. In the heating mode, the changes would be initiated by the conversion of the stable low-temperature state to the intermediate state at $T_{gg}$. At that temperature, the intermediate state would spontaneously convert to the liquid-crystalline state, since $T_{gg}$ is higher than $T_{gl}$. Similarly, in the cooling mode, changes would be initiated by the conversion of the liquid-crystalline state to the intermediate state at $T_{gl}$, at which temperature the intermediate should spontaneously convert to the stable low-temperature state, in the absence of kinetic limitations. Thus, for these shorter chain compounds, the above postulates predict that the necessary intermediate would be unstable with respect to one of the other two states at all temperatures, and DSC should report apparently single phase changes which nevertheless incorporate both the gel/gel transition and the gel/liquid-crystalline phase transition at $T_{gg}$ and $T_{gl}$ in the heating and cooling modes, respectively.

There are some interesting aspects of the above postulates with regard to the interpretation of the data described here. First, they suggest that the thermotropic behavior of the shorter chain compounds would be triggered by different events in the heating and cooling modes. In the heating mode, the "trigger

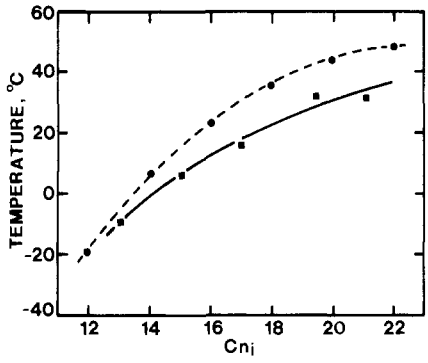


FIGURE 7: Chain-length dependence of the projected gel/gel transition temperatures of the even-numbered (●) and odd-numbered (■) 1,2-diisoacyl-PC's.

event" would be the gel/gel transition, while the conversion from a disordered acyl chain conformation to a predominantly all-trans acyl chain conformation (i.e., the gel/liquid-crystalline phase transition) would trigger the events in the cooling mode. Consequently, the heating endotherms of these shorter chain compounds might be expected to have some features in common with the gel/gel transitions resolved for the longer chain members. This is supported by our observations, since the heating endotherms of the shorter chain PC's were found to be subject to hysteresis and sensitive to the presence of ethylene glycol in the aqueous phase, as are the gel/gel transitions resolved for the longer chain compounds. In addition, these considerations also suggest that the characteristic temperatures of the cooling exotherms of the shorter chain PC's are better indicators of their "real" gel/liquid-crystalline phase transition temperatures than are the characteristic temperatures of their heating endotherms. Thus, the smooth curve drawn in Figure 4B could be interpreted as evidence for the absence of any odd/even alternation with respect to the chain-length dependence of hydrocarbon chain melting in this series of isoacyl-PC's. In this respect, the behavior of these compounds would be similar to that reported for the saturated unbranced PC's (Silvius et al., 1979) and would suggest that the gel/liquid-crystalline phase transition may be a simple chain-melting phenomenon like the melting of linear saturated paraffins (Malkin, 1952).

Second, these considerations also suggest that the characteristic temperatures of the heating endotherms of the shorter chain compounds are actually indicators of their gel/gel transition temperatures. Hence, those apparent gel/gel transition temperatures were plotted as a function of acyl chain number in Figure 7, along with the $T_{gg}$ values resolved for the longer chain compounds. The figure shows that the $T_{gg}$ values projected for the shorter chain compounds fit in very well with the odd/even alternation characteristic of the gel/gel transition values, in spite of the uncertainties in the accuracy of the $T_{gg}$ values resolved for the 19i and 21i PC's. Odd/even alternation is generally considered to be a solid-state phenomenon associated with differences in the packing modes and/or hydrocarbon chain tilt angles of compounds which have large groups substituted at the end(s) of long hydrocarbon chains (Malkin, 1952; Von Sydow, 1956). Thus, the odd/even alternation in the gel/gel transition temperatures, along with the observation that the range of enthalpies characteristic of the gel/gel transitions of the even-numbered members is significantly higher than that of the odd-numbered members of the series, may be indicative of tilting of the acyl chains in the stable conformation at temperatures below $T_{gg}$ and of significant differences in the acyl chain packing modes of the odd- and even-numbered isoacyl-PC's at those temperatures. Furthermore, the absence of odd/even alternation in the projected gel/liquid-crystalline phase transition temperatures may also be indicative of an acyl chain conformation normal to the bilayer plane in the gel-state conformation postulated to be the intermediate from which the liquid-crystalline state is formed.

With the above postulates, the parameters characteristic of the thermotropic phase behavior of these isoacyl-PC's can be tentatively assigned as shown in Table II. The assigned values for the gel/liquid-crystalline phase transition temperatures and the associated enthalpy changes are lower than those determined for the linear saturated acyl chain PC's of similar carbon number, an observation which may be indicative of differences in the acyl chain packing modes of the respective gel-state conformations from which the liquid-crystalline state is formed. We have proposed that a loosely packed gel-state conformation with fully extended acyl chains could function as the intermediate necessary for the interconversion between the stable low-temperature conformation and the liquid-crystalline state. The existence of such a loosely packed gel state has been suggested by a monolayer study which showed that in the liquid-condensed state the molecular area of branched-chain PC's is greater than that of unbranched saturated PC's (Kannenberg et al., 1983). Furthermore, a number of studies on bacterial membranes, which have been enriched with branched fatty acyl chains, have suggested that methyl-branched acyl chains are more loosely packed than are unbranched chains [see Kannenberg et al. (1983) for a discussion and references]. Our observations and those presented earlier (Silvius & McElhaney, 1979) also show that the gel/liquid-crystalline phase transition temperatures of these PC's occur at temperatures some 20 °C lower than those of the saturated chain compounds of equal acyl chain number, even though this is not reflected on a comparable lowering of the melting point of the free fatty acids (Gunstone, 1968). The existence of a loosely packed gel-state intermediate as the precursor for the formation of the liquid-crystalline state could explain these observations.

Another of the features of the "model" described is the prohibition of direct interconversions betwen the stable low-temperature conformation and the liquid-crystalline state. Given that in the stable low-temperature conformation the acyl chains would certainly be constrained so as to accommodate the seemingly incompatible requirements for polar interactions at the bilayer/solvent interface, interchain van der Waals interactions, and the steric accommodation of the methyl iso branch, the above restriction may not be unreasonable since it can be argued that the entropy loss in converting from a disordered liquid-crystalline state to a conformation subject to the above constraints may be too large to be accomplished in one step. Furthermore, the chain-packing conformation which best accommodates the above requirements would certainly reduce the motional freedom of the methylene groups, and chain melting may not be feasible while the chains remain in that conformation. X-ray diffraction studies on crystalline $C_{16i}$ and $C_{19i}$ fatty acids (Stenhagen et al., 1952; Abrahamsson, 1959a) have shown that in the solid state the acyl chains are packed at a greater angle of tilt to the bilayer normal than in crystalline straight-chain fatty acids. This has been ascribed to the necessity of accommodating the methyl branch in the space between the methyl end groups while maintaining the interchain van der Waals interactions and polar interactions between the carboxyl groups (Abrahamsson, 1959b). In the stable low-temperature conformation, the packing of the iso-branched acyl chains of the PC's is probably subject to similar

constraints, which may be compounded by the conformational nonequivalence reported for the *sn*-1 and *sn*-2 acyl chains of symmetric-chain PC's (Pearson & Pascher, 1979). Thus, it is feasible that in the stable, low-temperature conformation, the added constraints necessary for the accommodation of the methyl branch of the conformationally shorter *sn*-2 acyl chain may further reduce the motional freedom of the methylene segments of the acyl chains and effectively prevent the onset of trans/gauche isomerism (i.e., the formation of the liquid-crystalline state) from that packing conformation.

## ACKNOWLEDGMENTS

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**Registry No.** $C_{12i}$, 2724-56-3; $C_{13i}$, 5681-98-1; $C_{14i}$, 2724-57-4; $C_{15i}$, 2485-71-4; $C_{16i}$, 4669-02-7; $C_{17i}$, 1603-03-8; $C_{18i}$, 2724-58-5; $C_{19i}$, 2724-59-6; $C_{20i}$, 6250-72-2; $C_{21i}$, 59708-73-5; $C_{22i}$, 6704-01-4; $MeC_{12i}$, 5129-56-6; $MeC_{13i}$, 5129-57-7; $MeC_{14i}$, 5129-58-8; $MeC_{15i}$, 5129-59-9; $MeC_{16i}$, 5129-60-2; $MeC_{17i}$, 6929-04-0; $MeC_{18i}$, 5129-61-3; $MeC_{19i}$, 55124-97-5; $MeC_{20i}$, 65301-91-9; $MeC_{21i}$, 95799-86-3; $MeC_{22i}$, 91183-91-4; 12iPC, 71368-26-8; 13iPC, 71368-25-7; 14iPC, 71368-24-6; 15iPC, 71368-23-5; 16iPC, 71368-22-4; 17iPC, 71368-21-3; 18iPC, 60683-79-6; 19iPC, 95799-74-9; 20iPC, 95799-75-0; 21iPC, 95841-34-2; 22iPC, 95841-35-3; $HO_2C(CH_2)_8CO_2H$, 111-20-6; $HO_2C(CH_2)_{10}CO_2H$, 693-23-2; $HO_2(CH_2)_{11}CO_2H$, 505-52-2; $HO_2C(CH_2)_{12}CO_2H$, 821-38-5; $MeO_2C(CH_2)_8CO_2Me$, 106-79-6; $MeO_2C(CH_2)_{10}CO_2Me$, 1731-79-9; $MeO_2C(CH_2)_{11}CO_2Me$, 1472-87-3; $MeO_2C(CH_2)_{12}CO_2Me$, 5024-21-5; $HO_2C(CH_2)_4CO_2Me$, 627-91-8; $HO_2C(CH_2)_7CO_2Me$, 2104-19-0; $HO_2C(CH_2)_8CO_2Me$, 818-88-2; $HO_2C(CH_2)_{10}CO_2Me$, 3903-40-0; $HO_2C(CH_2)_{11}CO_2Me$, 3927-59-1; $HO_2C(CH_2)_{12}CO_2Me$, 50515-99-6; $ClC(O)(CH_2)_4CO_2Me$, 111-50-2; $ClC(O)(CH_2)_7CO_2Me$, 123-98-8; $ClC(O)(CH_2)_8CO_2Me$, 111-19-3; $ClC(O)(CH_2)_{10}CO_2Me$, 4834-98-4; $ClC(O)(CH_2)_{11}CO_2Me$, 35691-43-1; $ClC(O)(CH_2)_{12}CO_2Me$, 21646-49-1; $CH_3CH(CH_3)(CH_2)_3CO(CH_2)_4CO_2Me$, 95799-76-1; $CH_3CH(CH_3)CH_2CO(CH_2)_7CO_2Me$, 95799-77-2; $CH_3CH(CH_3)CH_2CO(CH_2)_8CO_2Me$, 95799-78-3; $CH_3CH(CH_3)(CH_2)_2CO(CH_2)_8CO_2Me$, 95799-79-4; $CH_3CH(CH_3)(CH_2)_3CO(CH_2)_8Co_2Me$, 95799-80-7; $CH_3CH(CH_3)(CH_2)_2CO(CH_2)_{10}CO_2Me$, 95799-81-8; $CH_3CH(CH_3)(CH_2)_3CO(CH_2)_{10}CO_2Me$, 95799-82-9; $CH_3CH(CH_3)(CH_2)_3CO(CH_2)_{11}CO_2Me$, 95799-83-0; $CH_3CH(CH_3)(CH_2)_3CO(CH_2)_{12}CO_2Me$, 95799-84-1; $CH_3CH(CH_3)(CH_2)_4CO(CH_2)_{12}CO_2Me$, 95799-85-2; $CH_3CH(CH_3)(CH_2)_5CO(CH_2)_{12}CO_2Me$, 95841-36-4; 1-bromo-4-methylpentane, 626-88-0; 1-bromo-2-methylpropane, 78-77-3; 1-bromo-3-methylbutane, 107-82-4; 1-bromo-5-methylhexane, 35354-37-1; 1-bromo-6-methylheptane, 52648-04-1; ethylene glycol, 107-21-1.

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