Biochemistry **1985**, *24*, 4903–4911

Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing ω-Cyclohexyl Fatty Acids. Differential Scanning Calorimetric and ^{31}P NMR Spectroscopic Studies[†]

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Received January 7, 1985

ABSTRACT: The thermotropic phase behavior of aqueous dispersions of 10 phosphatidylcholines containing ω-cyclohexyl-substituted acyl chains was studied by differential scanning calorimetry and ^{31}P nuclear magnetic resonance spectroscopy. The presence of the ω-cyclohexyl group has a profound effect on the thermotropic phase behavior of these compounds in a manner dependent on whether the fatty acyl chains have odd- or even-numbered linear carbon segments. The thermotropic phase behavior of the odd-numbered phosphatidylcholines is characterized by a single heating endotherm that was shown to be a superposition of at least two structural events by calorimetric cooling experiments. ^{31}P NMR spectroscopy also showed that the single endotherm of the odd-chain compounds is the structural equivalent of a concomitant gel–gel and gel to liquid-crystalline phase transition. The calorimetric behavior of the even-numbered phosphatidylcholines is characterized by a complex array of gel-state phenomena, in addition to the chain-melting transition, in both the heating and cooling modes. The gel states of these even-numbered compounds are characterized by a relatively greater mobility of the phosphate head group as seen by ^{31}P NMR spectroscopy. The differences between the odd-numbered and even-numbered compounds are reflected in a pronounced odd–even alternation in the characteristic transition temperatures and enthalpies and in differences in their responses to changes in the composition of the bulk aqueous phase. Moreover, both the odd-numbered and even-numbered ω-cyclohexylphosphatidylcholines exhibit significantly lower chain-melting transition temperatures and enthalpies than do linear saturated phosphatidylcholines of comparable chain length. These observations suggest that the presence of the ω-cyclohexyl ring in the fatty acyl chains of a phosphatidylcholine bilayer results in a considerable disruption of organization of both the polar head groups and the hydrocarbon chains and that these disruptions are more pronounced in the even-numbered compounds.

Fatty acids containing bulky substituents near their methyl termini are fairly common constituents of the membrane lipids of bacterial microorganisms. Of those, the ω-cyclohexyl fatty acids are of considerable interest, since they are the predominant acyl chains (≃70 mol %) in the membrane lipids of several strains of the acidothermophilic bacterium *Bacillus acidocaldarius* (DeRosa et al., 1971, 1972; Oshima & Ariga, 1975; Blume et al., 1978) and the bacteriophage ϕNS11 which infects it (Sakai et al., 1979). These fatty acids have also been found as significant components of the membrane lipids of the mesophilic bacterium *Curtobacterium pusillum* (Suzuki et al., 1981). In addition, some ω-cyclohexyl fatty acids are able to support normal growth of the mesophilic, cell wall-less microorganism *Acholeplasma laidlawii* B which has been made fatty acid auxotrophic, in which case the ω-cyclohexyl fatty acid is essentially the only fatty acyl group present in the membrane lipids (unpublished observations from this laboratory). This latter observation is very interesting, since it shows that these fatty acids possess all of the structural and physical properties necessary to maintain normal membrane function in this organism and that these fatty acids can support growth under conditions less extreme than those preferred by *B. acidocaldarius*.

In principle, the presence of cyclohexane rings in the middle of the lipid bilayer of a biomembrane should present some unique and interesting problems with regard to the acyl chain packing modes needed to accommodate its bulk and rigidity, while the structural and dynamic properties which make the membrane biologically viable are maintained. Most of the current data on acyl chain structure and membrane function have been derived from studies on linear saturated and cis-monounsaturated acyl chain systems, from which extrapolations can be made. However, such extrapolations may not be very reliable, since some studies on model membranes containing methyl iso-branched fatty acyl chains have shown that the branching of an acyl chain can have profound effects on the thermotropic phase behavior of such systems (Lewis & McElhaney, 1985; Mantsch et al., 1985). Some studies on model membranes composed of phosphatidylcholines (PC's)[1] containing ω-cyclohexyl fatty acyl chains have shown that the presence of an ω-cyclohexyl ring results in a lowering of the gel–liquid-crystalline phase transition temperature when compared with PC's containing linear saturated fatty acids (Endo et al., 1982; Kannenberg et al., 1984) and have hinted at potential miscibility problems between the straight-chain and ω-cyclohexyl-chain phospholipids (Endo et al., 1982). Other studies on model systems have shown that membrane vesicles containing ω-cyclohexyl fatty acyl structures are very resistant to permeation by small molecules, and have suggested that such a property may be decisive in conferring biological viability to these structures under the extreme conditions favoring the survival of the acidothermophilic bacterium in which

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[†] This work was supported by Operating and Major Equipment Grants from the Medical Research Council of Canada and by Major Equipment Grants from the Alberta Heritage Foundation for Medical Research.

[1] Abbreviations: DSC, differential scanning calorimetry; PC, phosphatidylcholine; NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

0006-2960/85/0424-4903$01.50/0

Table I: Precursors to the Synthesis of ω-Cyclohexyl Fatty Acids

fatty acid[a]	carboxyl fragment	alkyl-terminal fragment
$\omega_{cy}C_9$	adipic acid monomethyl ester[b]	1-bromo-3-cyclohexylpropane
$\omega_{cy}C_{10}$	azelaic acid monomethyl ester[b]	cyclohexylmethyl bromide
$\omega_{cy}C_{11}$	1,10-decanedioic acid	cyclohexylmethyl bromide
$\omega_{cy}C_{12}$	1,10-decanedioic acid	1-bromo-2-cyclohexylethane
$\omega_{cy}C_{13}$	1,12-dodecanedioic acid	cyclohexylmethyl bromide
$\omega_{cy}C_{14}$	1,12-dodecanedioic acid	1-bromo-2-cyclohexylethane
$\omega_{cy}C_{15}$	1,14-tetradecanedioic acid	cyclohexylmethyl bromide
$\omega_{cy}C_{16}$	1,14-tetradecanedioic acid	1-bromo-2-cyclohexylethane
$\omega_{cy}C_{17}$	1,13-tridecanedioic acid	1-bromo-4-cyclohexylbutane
$\omega_{cy}C_{18}$	1,14-tetradecanedioic acid	1-bromo-4-cyclohexylbutane

[a] The fatty acids are designated by the notation $\omega_{cy}C_n$. The prefix ω denotes the ω-cyclohexyl group and n denotes the length of the carbon chain to which the ring is attached. [b] These monomethyl esters were obtained from Aldrich Chemical Co.

they naturally occur (Sumamoto et al., 1982; Benz et al., 1983; Kannenberg et al., 1984). However, at this time such judgments must be considered speculative in view of the limited number of studies on membrane systems containing the ω-cyclohexyl hydrocarbon chain. Consequently, we have synthesized a series of ω-cyclohexyl fatty acids and their respective phosphatidylcholines for a thorough study of the effect of this substituent on the structure and function of model and biological membranes. The results of some DSC and ^{31}P NMR spectroscopic studies of aqueous dispersions of this series of ω-cyclohexyl-PC bilayers are presented here.

MATERIALS AND METHODS

The sources of commercially available chemicals, solvents, and chromatographic adsorbents and their pretreatment and/or purification were the same as previously used in this laboratory (Lewis & McElhaney, 1985). 1-Bromo-2-cyclohexylethane was synthesized from cyclohexanemethanol (Aldrich) by the chain elongation procedure used by Silvius & McElhaney (1980). 1-Bromo-3-cyclohexylpropane was synthesized from cyclohexylmethyl bromide using the ethylene oxide elongation procedure used by Lewis & McElhaney (1985). 1-Bromo-4-cyclohexylbutane was synthesized from cyclohexanebutyric acid (Aldrich) by the bromination of the alcohol formed after reduction of the acid with lithium aluminum hydride. The ω-cyclohexyl fatty acids were synthesized from the precursors listed in Table I by the chemical reactions used by Lewis & McElhaney (1985) and were recrystallized from 70% ethanol ($n \leq 15$) or hexane–chloroform (9:1 v/v) ($n \geq 15$). The procedures for synthesis and purification of the PC's used in this study were the same as those used for the preparation of highly purified samples of 1,2-diisoacylphosphatidylcholines (Lewis & McElhaney, 1985).

The thermotropic phase behavior of fully hydrated samples of these phospholipids was studied in a Microcal MC-1 high-sensitivity differential scanning calorimeter and a Perkin-Elmer DSC-2C differential scanning calorimeter equipped with a thermal analysis data station. The methods of sample preparation and quantification were the same as previously used in this laboratory (Lewis & McElhaney, 1985). ^{31}P NMR spectra were recorded on a Bruker XHS-270 NMR spectrometer. The details of sample preparation and data acquisition parameters have been described previously (Mantsch et al., 1985).

RESULTS

Thermotropic Phase Behavior in Water. 1,2-Diacyl-PC's containing ω-cyclohexyl acyl chains of 9–18 linear carbon segments (15–24 total carbon atoms) were synthesized from the corresponding fatty acids, and the thermotropic phase behavior of aqueous multilamellar dispersions of these lipids was studied by DSC. The compounds that had linear carbon segments of more than 10 carbons exhibited phase transitions above 0 °C and were studied with the high-sensitivity calorimeter, while the others were characterized by the lower sensitivity Perkin-Elmer DSC-2C instrument. Figure 1 shows the heating endotherms resolved by the high-sensitivity instrument. It is clear that there are profound differences in the thermotropic phase behavior of those compounds that had odd- and even-numbered linear carbon segments, a trend that is also characteristic of the short-chain compounds (n = 9, 10) studied with the low-sensitivity instrument (see Figure 2). Those compounds with odd-numbered linear carbon segments exhibit heating endotherms consisting of a single sharp transition that progressively broadens with the increasing length of the linear chain segment. These transitions show a modest hysteresis, since there are small increases in the transition temperatures and widths with increasing heating rates. The endotherms presented in Figure 1 were recorded at scan rates which are sufficiently slow so as to avoid such kinetic distortions. Infrared spectroscopic studies show that, in addition to the chain-melting transition, those single endotherms include structural changes normally associated with gel-state packing transitions (Mantsch et al., unpublished observations). In contrast, those compounds with even-numbered linear carbon segments exhibit heating endotherms consisting of at least two broad, lower temperature transitions and one sharp, high-temperature transition. The latter was shown to include the

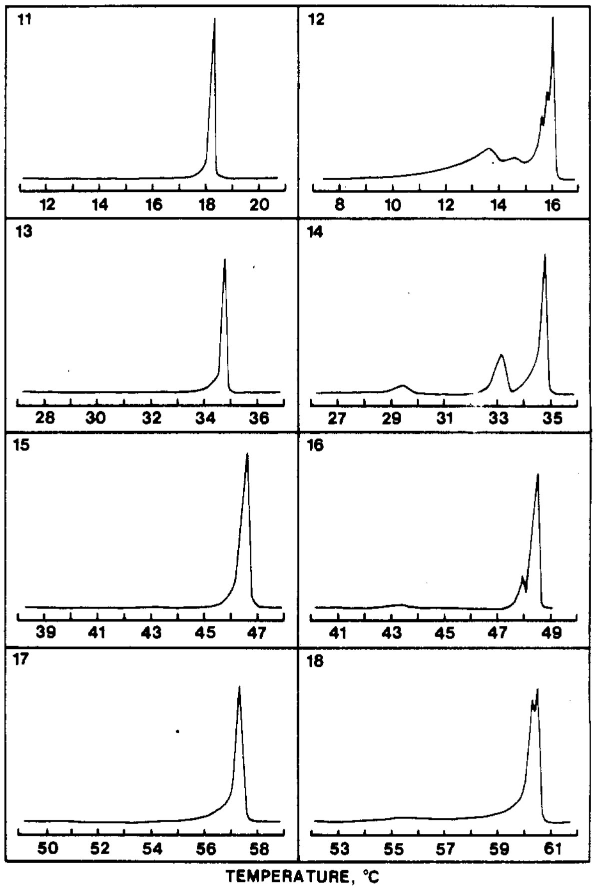

FIGURE 1: High-sensitivity DSC heating thermograms of 1,2-di-ω-cyclohexyl-PC's of $n \geq 11$. The thermograms were obtained at the following heating rates: $C_{11} \simeq 18$ °C h^{-1}; $C_{12} \simeq 9$ °C h^{-1}; $C_{13} \simeq 18$ °C h^{-1}; $C_{14} \simeq 6$ °C h^{-1}; $C_{15} \simeq 18$ °C h^{-1}; $C_{16} \simeq 6$ °C h^{-1}; $C_{17} \simeq 14$ °C h^{-1}; $C_{18} \simeq 7$ °C h^{-1};

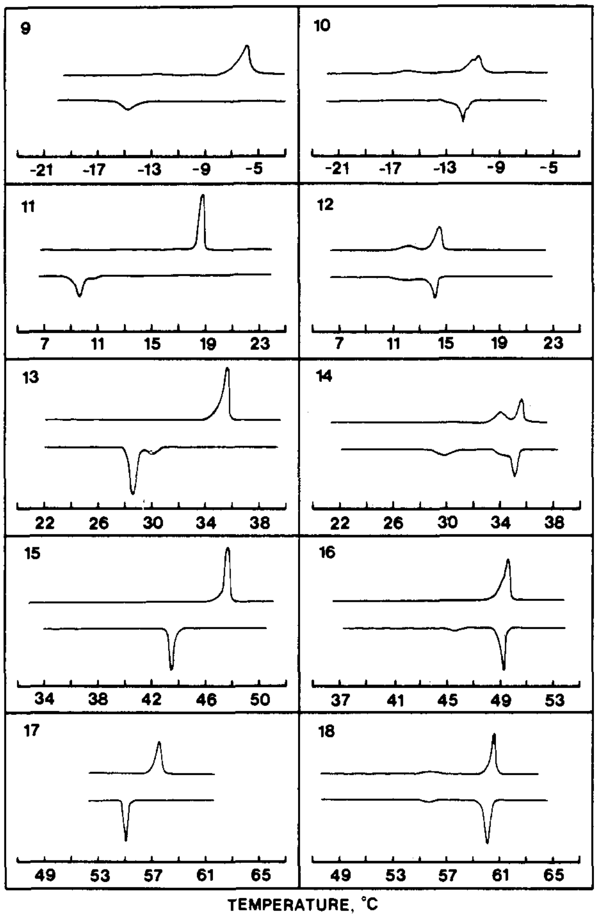

FIGURE 2: Low-sensitivity DSC heating and cooling thermograms of aqueous dispersions of the 1,2-di-ω-cyclohexyl-PC's. The thermograms were all obtained at a scan rate of 18.75 °C h^{-1}.

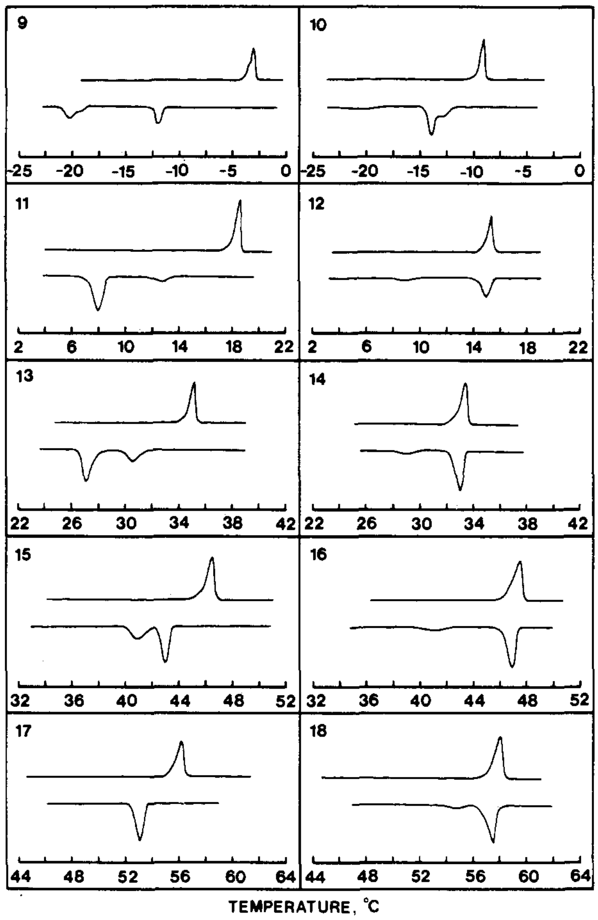

FIGURE 3: Low-sensitivity DSC heating and cooling thermograms of dispersions of the 1,2-di-ω-cyclohexyl-PC's in aqueous ethylene glycol. The thermograms were all obtained at a scan rate of 18.75 °C h^{-1}.

chain-melting transition by infrared spectroscopic studies, which also assigned the lower temperature events to gel-state packing rearrangements (Mantsch et al., unpublished observations). The gel-state transitions exhibit a marked heating hysteresis, and resolution from the chain-melting transition to the extent shown in Figure 1 was only feasible using very slow heating rates (5–9 °C h^{-1}). The lower temperature, gel-state event contributes a progressively smaller fraction of the total enthalpy change as the length of the linear carbon segment increases.

The cooling exotherms of the above compounds were recorded on the Perkin-Elmer DSC-2C instrument at the slowest scan rate feasible with that instrument (18.75 °C h^{-1}). Those exotherms are shown in Figure 2 along with the corresponding heating endotherms recorded on the same instrument. In spite of the lower resolution afforded by the DSC-2C, it is clear that there are significant differences in the cooling behavior of the ω-cyclohexyl-PC's with odd- and even-numbered linear carbon segments. For those compounds with odd-numbered chains, the reversal temperatures of the cooling exotherms are some 5–10 °C lower than those of their corresponding heating endotherms. This appears to be an intrinsic property of these PC's and not an experimentally induced artifact, since the observed behavior is dependent only upon the thermal history of the sample and is unaffected by prolonged annealing at temperatures between those of the heating and cooling peaks. In addition, the cooling exotherms of these odd-numbered PC's show evidence of a two-step conversion, especially in the case of the shorter chain compounds (n = 11, 13), which show a prominent high-temperature shoulder. The contribution of this shoulder to the total enthalpy change decreases with increasing acyl chain length and is not detectable in the case of the longer chain compounds (n = 15, 17). In contrast, the PC's with even-numbered linear carbon segments exhibit a simpler cooling behavior, since the characteristic temperatures of the higher temperature transitions coincide in both the heating and cooling modes. In addition, their gel-state events show a marked cooling hysteresis, since they tend to be broader and to occur at lower temperatures than when observed in the heating mode. The reversibility characteristics of the gel-state transitions of these even-numbered PC's were unlike those of their odd-numbered counterparts. In contrast to the odd-numbered PC's, the heating–cooling reversibility characteristics of the gel-state transitions of the even-numbered compounds are the result of the slow rates of attainment of equilibrium, and as such are dependent on scan rates and, at equilibrium, are independent of the thermal history of the sample.

Thermotropic Phase Behavior in Aqueous Ethylene Glycol. These studies were prompted by the common use of aqueous ethylene glycol as the bulk aqueous phase, in order to prevent ice formation, when DSC studies are carried out below 0 °C, and by a study describing a perturbation of the gel-state behavior of methyl iso-branched fatty acyl-containing PC's by ethylene glycol (Lewis & McElhaney, 1985). Illustrated in Figure 3 are the heating endotherms and cooling exotherms (as recorded with the Perkin-Elmer calorimeter) of this series of ω-cyclohexyl-PC's dispersed in 50% aqueous ethylene glycol (v/v). It is clear that the thermotropic phase behavior of all the compounds studied is affected by the change in bulk aqueous phase in a manner dependent on whether they contain odd- or even-numbered linear hydrocarbon segments. For those compounds with odd-numbered chains, the heating behavior in aqueous ethylene glycol is indistinguishable from that observed in water. The cooling behavior of the odd-numbered PC's also resembles that of an aqueous dispersion, in that the thermal events occur at temperatures lower than those characteristic of the heating endotherms, and in general consist of two thermotropic events, in sharp contrast to the single endotherms observed in the heating mode. However, unlike the aqueous dispersion, there is a better resolution of the two thermal events on the temperature scale, and in general the contribution of the lower temperature event to the total enthalpy change decreases with increasing length of the linear carbon segment and is not observable for the C_{17} species.

In contrast to the odd-numbered compounds, the effect of ethylene glycol on the even-numbered PC's is mainly observed in the heating mode. Their cooling behavior consists of two thermotropic events that tend toward convergence on the temperature scale with increasing length of the linear carbon chain, just as observed with aqueous dispersions. The characteristic temperature of the higher temperature event coincides with that of the heating endotherm, while the lower temperature event shows evidence of cooling hysteresis, comparable to that observed for vesicles in water alone. However, unlike the aqueous dispersions, the heating behavior of these even-numbered ω-cyclohexyl-PC's consists of a single heating endotherm, which occurs at temperatures close to those of the chain-melting transition observed with the aqueous dispersions. In addition, these endotherms show a modest heating hysteresis comparable to that observed for aqueous dispersions of the odd-numbered ω-cyclohexyl-PC's studied.

Another interesting feature of these studies is the observation that the behavior of the short-chain ω-cyclohexyl-PC's (n = 9, 10) in aqueous ethylene glycol is anomalous with respect to the trends set by the longer chain members ($n \geq 11$). For the C_9 species, the heating behavior in aqueous ethylene glycol consists of a heating endotherm with a pronounced low-temperature shoulder; i.e., there is evidence of two thermal events in the heating mode, in sharp contrast to the single endotherm observed for an aqueous dispersion of this compound. The cooling behavior of the C_9 species in aqueous ethylene glycol resembles that of the longer chain, odd-numbered members in that two cooling exotherms are observed at temperatures lower than that of the heating endotherm. However, the low-temperature exotherm of this compound has a pronounced shoulder, suggesting that its cooling behavior actually consists of three thermotropic events, in contrast to the two events resolved for the longer chain, odd-number members dispersed in the same solvent. For the C_{10} species, its heating behavior in aqueous ethylene glycol resembles that of the longer chain, even-numbered compounds in that a single heating endotherm is found in the same solvent. However, its cooling behavior differs in that three thermotropic events can be resolved at temperatures lower than that of the endotherm observed in the heating mode. Finally, the anomalous behavior of these short-chain ω-cyclohexyl-PC's in aqueous ethylene glycol is also apparent from the characteristic enthalpy values, which are up to 3 times greater than those characteristic of an aqueous dispersion, in sharp contrast to the longer chain members (see below).

Table II: Thermodynamic Characterization of the Heating Endotherms of the 1,2-Di-ω-cyclohexyl-PC's

PC	$T_m(H_2O)^a$ (°C)	$T_m(E.G.)^a$ (°C)	ΔH^c (kcal mol^{-1})
C_9	−6.8	−3.3	4.4 (6.7)
C_{10}	−10.8	−9.3	1.2 (4.7)
C_{11}	18.3	18.4	11.7
C_{12}	16.0	15.5	6.3
C_{13}	34.9	34.8	13.2
C_{14}	34.6	33.4	7.8
C_{15}	46.6	46.6	16.3
C_{16}	48.5	47.3	8.7
C_{17}	57.6	56.2	$\geq 18^b$
C_{18}	60.6	57.9	$\geq 11^b$

[a] Refers to the bulk solvent phase. H_2O = water; E.G. = 50% aqueous ethylene glycol (v/v). [b] Hydrolysis of these samples during the DSC experiment resulted in a diminution of the measured transition enthalpy by 2–5% on each successive run. The listed values were obtained from the first runs of several samples and should be considered minimum values. [c] The ΔH values quoted are the total enthalpy changes measured by both calorimeters used (where applicable) and have an intrinsic error of ±3%. The values in parentheses are listed for dispersions in aqueous ethylene glycol where those values differed significantly from those of aqueous dispersions.

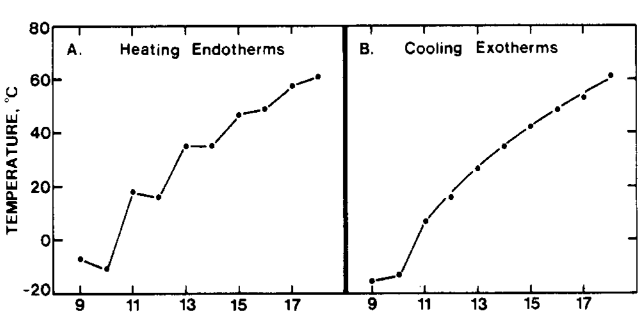

FIGURE 4: (A) Chain length dependence of the characteristic temperatures of the main heating endotherms of the 1,2-di-ω-cyclohexyl-PC's. (B) Chain length dependence of the characteristic temperatures of the main cooling exotherms of the 1,2-di-ω-cyclohexyl-PC's.

Thermodynamic Characterization. The total enthalpy changes characteristic of the thermotropic phase behavior of these ω-cyclohexyl-PC's are listed in Table II, along with the reversal temperatures of the main heating endotherms. The enthalpy values listed are the same whether measured in the heating or cooling modes. No attempt was made to subdivide the transition enthalpy of the even-numbered PC's into their constituent parts, since, in general, there was incomplete resolution of the various thermotropic events on the temperature scale, and some of these events have not yet been assigned to any defined physical process. This, in addition to the fact that the comparable phenomena of the odd-numbered compounds are not separated on the temperature scale (see Discussion), has precluded a thorough analysis of the various thermodynamic parameters. Nevertheless, it is clear that despite the apparently simpler heating behavior of the odd-numbered PC's, their range of characteristic enthalpy values is higher than that of their even-numbered counterparts of comparable carbon number. It is also clear that the effects of aqueous ethylene glycol on the thermotropic phase behavior of these compounds is not reflected in the transition enthalpies of the longer chain compounds ($n \geq 11$). The transition enthalpies of aqueous dispersions of the short-chain PC's were considerably lower than those of dispersions in aqueous ethylene glycol. This, and some aspects of the thermotropic phase behavior of these short-chain compounds, is anomalous with respect to trends set by their longer chain counterparts.

Table II also lists the characteristic temperatures of the main heating endotherms of the entire series of ω-cyclohexyl-PC's studied. With the probable exception of the C_9 species, these temperatures appear not to have been severely affected by the presence of ethylene glycol in the bulk aqueous phase. These transition temperatures are plotted as a function of the number

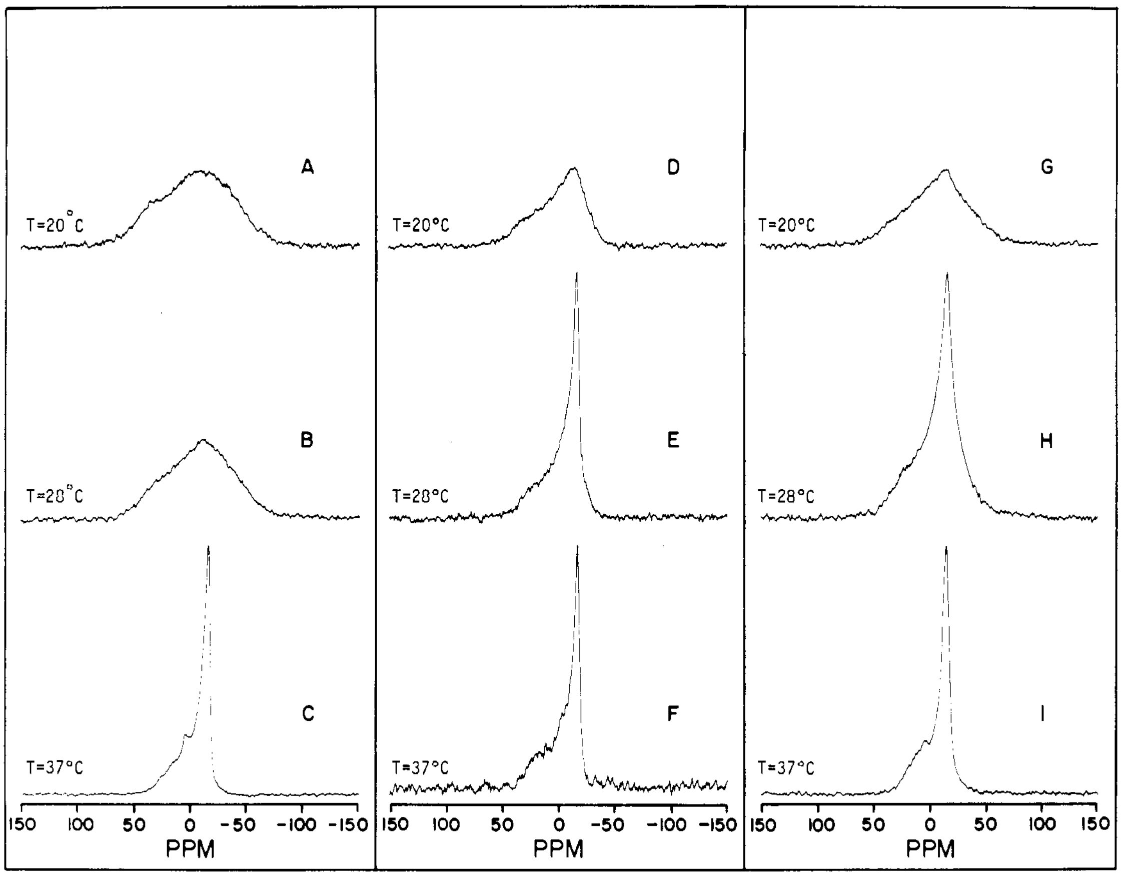

FIGURE 5: Proton-decoupled ^{31}P NMR spectra of dispersions of (i) 13-cyclohexyl-PC in water (spectra A–C), (ii) 14-cyclohexyl-PC in water (spectra D–F), and (iii) 14-cyclohexyl-PC in aqueous ethylene glycol (spectra G–I). The data were acquired at the temperature indicated in a temperature sequence that corresponded to the heating mode of the DSC experiment.

of carbon atoms in the linear chain segments in Figure 4A (main heating endotherm temperatures were chosen because infrared spectroscopic studies have shown that hydrocarbon chain melting occurs at those temperatures). There is a pronounced odd–even alteration in these temperatures, comparable to that described for the melting points of the free fatty acids (Ishizawa et al., 1968a,b). However, unlike the free fatty acids, the magnitude of the odd–even discontinuities *decrease* with increasing chain length. Figure 4B shows the reversal temperatures of the main cooling exotherms plotted as a function of the number of linear carbons. The pronounced odd–even alternation characteristic of the heating endothermic transition temperatures is not observed for the longer chain PC's ($n \geq 11$). It is also apparent that the anomalous behavior of the short-chain compounds ($n = 9, 10$) is manifest here as a marked discontinuity from the smooth curve connecting these cooling exothermic transition temperatures.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra (see Figure 5) are presented for the C_{13} and C_{14} compounds, which are typical of the odd- and even-numbered ω-cyclohexyl-PC's studied. For the odd-numbered compounds, the low-temperature state is characterized by a broad spectrum (basal line width $\simeq$ 130 ppm; see spectra A and B) typical of slow, axially asymmetric motion on the ^{31}P NMR time scale (Seelig, 1978; Campbell et al., 1979), while at temperatures above the single heating endothermic transition, the spectral line shape approaches that which is typical of a phospholipid undergoing fast axially symmetric motion in a lipid bilayer in the liquid-crystalline state (see spectrum C). Similar spectral line shapes are also characteristic of dispersions of the odd-numbered PC's in aqueous ethylene glycol at temperatures below and above the single heating endothermic transition observed by DSC.

The spectra D and E illustrated in Figure 5 are of an aqueous dispersion of the C_{14} PC at temperatures (20 and 31 °C) which bracket the broad, low-temperature endotherm observed by DSC. At both temperatures the lipid was still in a gel-state conformation. The low-temperature gel state is characterized by a spectrum (basal line width $\simeq$ 90 ppm) that is narrower than that characteristic of the gel state of the odd-numbered compounds. In addition, the shape of the spectrum is characteristic of axially symmetric motion, though the width of the spectrum suggests that the motion of the phosphate group is relatively slow. It is also clear that while still in a gel-state conformation at 31 °C, the spectral line shape is similar to that of its liquid-crystalline state (see spectrum E). Thus, the limiting spectral line shape typical of fast axially symmetric motion in a bilayer structure is approached while the lipid is still in a gel-state conformation.

^{31}P NMR spectra were also recorded for dispersions of the C_{14} PC in aqueous ethylene glycol (see spectra G–I). Spectrum G acquired at 20 °C (13 °C below the DSC endothermic transition temperature) shows a broad, featureless spectral line shape (basal line width $\simeq$ 110 ppm broad), which is similar to but not identical with the "slow-motion spectra" characteristic of the gel state of the odd-numbered ω-cyclohexyl-PC's. This spectrum is slightly broader than that typical of an aqueous dispersion of that lipid at the same temperature and considerably narrower than that of a dispersion of the C_{13} PC in aqueous ethylene glycol at the same data acquisition temperature. In addition, spectrum G lacks the features characteristic of axially symmetric motion, in sharp contrast to the spectral properties of an aqueous dispersion of the compound at the same data acquisition temperature. At the higher temperatures there was a slight narrowing of the line width, and the gel-state approach to the endothermic transition temperature resulted in the appearance of a sharp peak near 20 ppm upfield, though the basal line width remained near 90 ppm. Spectrum H was acquired at 28 °C (5 °C below the endothermic transition temperature of the C_{14} PC in 50% aqueous ethylene glycol) and is atypical of any of the ^{31}P NMR spectra of aqueous dispersions of phospholipids. The spectrum acquired at 28 °C appears to exhibit features of both the gel-state spectrum G and the liquid-crystalline state spectrum I which was acquired at 37 °C. Simulations using a spectral addition program showed that if a slow rate of exchange between the gel and liquid-crystalline states is assumed, spectrum H could be constructed by a simple spectral addition, based on the further assumption that 25% of the lipids were in the liquid-crystalline state, or a state of similar ^{31}P NMR spectral characteristics, at the data acquisition temperature. This suggests that when the compounds were suspended in aqueous ethylene glycol, significant structural changes were occurring at temperatures that were well below that of the single endotherm resolved by DSC. Given the time required to acquire the spectrum ($\simeq$5 h), it is feasible that those changes occur on a time scale that is too slow to be resolved by DSC. This in turn would suggest that there is a significant kinetic component to the effects of ethylene glycol on the thermotropic phase behavior of the even-numbered ω-cyclohexyl-PC's.

DISCUSSION

A dominant feature of this work is the demonstration of a marked difference in the behavior of PC's containing ω-cyclohexyl fatty acids with odd- and even-numbered linear carbon segments. The difference was apparent in all aspects of this study and perhaps represents one of the more extreme examples of the so-called odd–even effect [see Malin (1952) and Von Sydow (1956)]. This effect is often observed in the solid-state behavior of a variety of long-chain paraffinic compounds and has been manifest in odd–even discontinuities in the melting points of several types of fatty acids (Malkin, 1952; Von Sydow, 1956; Silvius & McElhaney, 1979; Lewis & McElhaney, 1985), including some with ω-cyclohexyl substitutions (Ishizawa et al., 1968a,b, 1971). This behavior is generally considered to be a solid-state phenomenon that arises naturally from the differences in the possible end-group interactions of compounds which have odd- and even-numbered long paraffinic chains that are in a crystalline (or quasi-crystalline) lattice, with the long chains tilted to the end-group planes [see Broadhurst (1962)]. In fact, the tilting of the hydrocarbon chains to the end-group planes is dependent upon those end-group interaction effects, with the result that the odd- and even-numbered members of a homologous series of end-substituted long-chain paraffinic compounds tend to crystallize into forms with different chain-packing modes. Thus, odd–even discontinuities would be expected of all physical phenomena which are dependent upon the crystal structures of such compounds.

The odd–even effect would probably be exaggerated in the behavior of compounds that have bulky terminal substituents like a cyclohexane ring, especially in those compounds which also have a polar substituent at the opposite terminus (e.g., alcohols, fatty acids, etc.). The solid-state behavior of such compounds should be strongly influenced by the seemingly incompatible requirements for maximizing net polar interactions at the α end group and van der Waals interactions between the hydrocarbon chains, while accommodating the bulky ω end group. In the case of ω-cyclohexyl-substituted compounds, the above considerations may be further complicated by the relative rigidity of the cyclohexane ring. Our studies with molecular models have shown that the most stable all-trans conformations of an ω-cyclohexyl-substituted paraffinic compound occur when the cyclohexane ring adopts the chair conformation with the hydrocarbon chain in the equatorial position. In such a conformation three of the ring carbons would be continuous with the hydrocarbon chain zig-zag "line", with the others rigidly offset to one side. Thus, a dominant factor affecting the solid-state packing of the hydrocarbon chains should be the spatial requirements of those "side" carbons. Some X-ray studies on alcohols and fatty acids with ω-cyclohexyl substituents have suggested that those requirements might be met if the hydrocarbon chains were to adopt a tilt of approximately 60° to the normal to the end-group planes (Ishizawa, 1971a,b). This is in marked contrast to the near 30° tilt of the saturated, straight-chain compounds [see Muller (1927, 1928, 1930), Malin (1930) and Von Sydow (1956)]. The tendency to adopt such "extreme" hydrocarbon chain tilt is clearly the result of the packing requirements of the ω-cyclohexyl group and probably is the reason for the pronounced odd–even discontinuities in the melting points and long spacings of these compounds (Ishizawa et al., 1968a, b; Ishizawa, 1971a,b), as well as the odd–even discontinuities described in this study. In addition, single-crystal X-ray diffraction studies on the ω-cyclohexyl-substituted fatty acids (James et al., unpublished results) have shown that the odd- and even-numbered acids are characterized by radically different chain-packing parameters.

Given the above, the odd–even discontinuities in the behavior of these ω-cyclohexyl-PC's would be indicative of significant structural differences in their respective gel states. In particular, the data clearly show that, when compared with the odd-numbered compounds, the even-numbered members are characterized by a lower range of transition enthalpies and a greater mobility of the phosphate group in the gel state. These observations suggest that the even-numbered compounds are in a more loosely packed gel-state conformation than that of their odd-numbered counterparts. It is thus clear that, in this model membrane system, the accommodation of the ω-cyclohexyl end group is more favorable with the odd-numbered compounds studied. The fact that such odd–even discontinuities were observed implies that at least one of the gel-state, chain-packing conformations of either the even-numbered compounds or their odd-numbered counterparts must be tilted to the bilayer normal, since odd–even discontinuities would be very unlikely if the gel-state packing conformations were all normal to the bilayer plane. However, the details of the gel-state chain packing conformations of these compounds are outside the scope of the physical techniques used in this study and will only be defined when X-ray diffraction data become available. Nevertheless, there are some interesting structural implications of the ^{31}P NMR data, especially those of the even-numbered compounds. The data suggest that the gel-state conformations of the even-numbered compounds are sufficiently loose so as to allow the axially symmetric motion detected by ^{31}P NMR. The fact that, with the even-numbered compounds, the limiting ^{31}P NMR spectral line shape for fast axially symmetric motion in a phospholipid bilayer is approached before the hydrocarbon chain melts probably has further structural implications, since it seems unlikely that such phosphate head-group mobility would be feasible in any tightly packed gel-state conformation with the hydrocarbon chains tilted to the bilayer normal. Thus, it is probable that the gel-state conformations of these even-numbered ω-cyclohexyl-PC's which allow such phosphate mobility may be loosely packed, with the acyl chains normal to the bilayer plane.

The ω-cyclohexyl-PC's were also characterized by a pronounced odd–even alteration in the acyl-chain melting temperatures when measured on heating and, with the exception of the short-chain compounds ($n = 9, 10$), the absence of such alteration in the characteristic temperatures of the main cooling exotherms. This was directly attributable to the heating–cooling reversibility characteristics of the odd-numbered compounds, which followed a pattern similar to that described for some short-chain 1,2-diisoacyl-PC's for which the gel-state chain-packing phenomena and hydrocarbon-chain melting phenomena were not resolvable on the temperature scale (Lewis & McElhaney, 1985). The possibility of an inclusion of gel-state phenomena in the single heating endotherm characteristic of these odd-numbered PC's is supported by the relative complexity of their cooling behavior and by the ^{31}P NMR data. The latter clearly show that the change in the phosphate head-group mobilities concomitant with the single heating endothermic transitions of these odd-numbered compounds ranged from slow axially asymmetric motion to fast axially symmetric motion on the ^{31}P NMR time scale. The broad, slow-motion spectra characteristic of the gel state of the odd-numbered PC's are in general similar to those described for the sub-gel phase of DPPC (Fuldner, 1981; Lewis et al., 1984), as well as the stable, low-temperature gel states of the diisoacyl-PC's (Mantsch et al., 1985). Thus, in addition to hydrocarbon chain melting, the structural changes concomitant with the single heating endothermic transition of these odd-numbered compounds probably involve some gel-state phenomena, which may be similar in some respects to the gel-state transitions of the linear saturated PC's and the isoacyl-PC's. The concomitant occurrence of gel-state and chain-melting phenomena in the heating behavior of the odd-numbered compounds may provide a rationale for their anomalous heating–cooling reversibility characteristics. A similar trend was observed in the thermotropic behavior of the short-chain isoacyl-PC's, for which the reversal temperatures of their heating endotherms are higher than those of their cooling exotherms (Lewis & McElhaney, 1985). Such behavior was rationalized by suggesting that the formation of an intermediate is necessary for interconversions between the low-temperature gel state and the liquid-crystalline state and that the anomalous heating–cooling reversibility characteristics would occur if the temperature favoring interconversion between the intermediate and the stable gel state were higher than that favoring interconversion between the intermediate and liquid-crystalline state. Thus, in the case of the ω-cyclohexyl-PC's, there should be no anomalies in the heating–cooling reversibility characteristics of the even-numbered compounds (apart from some classical hysteresis effects), since the data described here clearly show that the gel-state packing modes likely to form the necessary intermediate occur at temperatures below those of the acyl chain melting transition. However, in the case of the odd-numbered PC's, their behavior would be interpreted as being consistent with a concomitant occurrence of both their gel-state and chain-melting phenomena at the gel–gel transition temperatures in the heating mode, and at their gel–liquid-crystalline phase transition temperatures in the cooling mode. As a result, the odd–even discontinuities in the heating endothermic transition of these ω-cyclohexyl-PC's would reflect an alternation between gel-state transition temperatures (odd-numbered compounds) and chain-melting transition temperatures (even-numbered compounds). In the cooling mode, there should be no odd–even discontinuities in the reversal temperatures of their main cooling exotherms, since those would occur at the gel–liquid-crystalline phase transition temperatures. With the exception of the short-chain PC's ($n = 9, 10$), which were anomalous with respect to the trends set by the longer chain compounds, the data described here are in general consistent with those predictions. However, it is also apparent from the cooling exotherms of the odd-numbered compounds that their cooling behavior was more complex than that of the short-chain isoacyl-PC's. In addition, the main cooling exotherms of these odd-numbered compounds, which according to the above rationale should occur at the gel–liquid-crystalline phase transition temperature, were not always the higher temperature event. Hence, it is clear that, despite the obvious similarities, the rationale described for the isoacyl-PC's does not accommodate all aspects of the behavior of these odd-numbered ω-cyclohexyl-PC's. Consequently, a full understanding of the thermotropic phase behavior of these compounds (both odd and even numbered) may only be feasible after the mechanistic details of their behavior in both the heating and cooling modes are defined.

Another interesting feature of this study is the sensitivity of the behavior of these ω-cyclohexyl-PC's to the composition of the bulk aqueous phase in a manner dependent on whether they contain odd- or even-numbered linear carbon segments. The effect of aqueous ethylene glycol on the behavior of the odd-numbered compounds was only observed in the cooling mode, where there appeared to be a better resolution of thermotropic events on the temperature scale and other quantitative differences. However, using the techniques described here, we were unable to assign the structural basis of those cooling exotherms, nor could we determine whether the observed differences were real or artifacts of kinetically limited processes. In the case of the even-numbered PC's, the effect of aqueous ethylene glycol was mainly observed in the heating mode, where we were unable to resolve the array of gel-state behavior characteristic of the aqueous dispersions. That such gel-state phenomena still occur was evident from the cooling behavior and the similarities in the transition enthalpies of dispersions of these even-numbered compounds in water and aqueous ethylene glycol. The ^{31}P NMR data indicate that, when compared with an aqueous dispersion, the mobility of their phosphate head group in the gel state is more restricted when dispersed in aqueous ethylene glycol. However, such "restrictions" do not translate into differences in transition enthalpies, and this suggests that they may be a reflection of properties extrinsic to these lipids, e.g., the higher viscosity of the aqueous ethylene glycol. In addition, the ^{31}P NMR data also suggest that there are structural changes occurring at temperatures below that of the single heating endotherm characteristic of dispersions of the even-numbered compounds in aqueous ethylene glycol and that those changes were occurring too slowly to be resolved by DSC at equilibrium. This shows that there is a significant kinetic component to the effects of aqueous ethylene glycol on the thermotropic phase behavior of the even-numbered ω-cyclohexyl-PC's, though the exact nature of such effects is still to be determined. Nevertheless there are some important implications of the solvent-mediated effects described here. First, these observations raise doubts as to the wisdom of using aqueous ethylene glycol as the bulk solvent phase for the characterization of the thermotropic phase behavior of lipids. The data reported here and elsewhere (Lewis & McElhaney, 1985) clearly show that the gel-state behavior of some lipids can be perturbed by such a solvent and suggest that great care in the choice of such solvent phases should be exercised. Second, the data described here also hint at a possible involvement of head-group and interfacial hydration changes in the thermotropic phase behavior of these lipids (both odd and even numbered). Since the inclusion of ethylene glycol in the bulk aqueous phase would be expected to reduce the polarity of the solvent phase and decrease the chemical activity of water, it seems reasonable to suggest that any hydration-dependent phase behavior of these lipids could be affected by the change in the properties of the solvent phase. We have found that aqueous ethylene glycol perturbs the gel-state behavior of the diisoacyl-PC's (Lewis & McElhaney, 1985), as well as the subtransition of the *n*-acyl-PC's (unpublished observations from this laboratory), and some infrared spectroscopic studies have suggested that hydration changes are involved in both processes (Cameron & Mantsch, 1982; Mantsch et al., 1984). Given this and the similarity of the above processes with some aspects of the behavior of these lipids as observed by ^{31}P NMR spectroscopy, the probable involvement of hydration changes in the thermotropic phase behavior of these ω-cyclohexyl-PC's cannot be ruled out.

Solvent-mediated effects are also apparent in the behavior of short-chain compounds ($n = 9, 10$), which are anomalous with respect to the trends set by their longer chain counterparts. The probability of hydration-dependent phenomena in the behavior of these lipids, and the fact that when in aqueous dispersion the bulk solvent phase is frozen at the temperatures at which they exhibit their thermotropic phase behavior, may account for some of the anomalies. However, it is clear that other considerations must be involved, since behavioral discontinuities between these short-chain compounds and their longer chain counterparts were observed when they were studied in aqueous ethylene glycol, which remained unfrozen throughout the entire temperature range investigated. Anomalies in the behavior of the shorter chain members of a homologous series of diacyl-PC's have been observed with the *n*-acyl series (Mabrey & Sturtevant, 1978; Silvius et al., 1979) and the isoacyl series (Lewis & McElhaney, 1985), and in both cases the enthalpies of chain melting were either measured or estimated to be small ($\leq$3 kcal mol^{-1}). Given that the contribution of the van der Waals interchain interactions to the overall free energy of stabilization of a lipid bilayer should decrease with decreasing chain length, it is possible that, for such short-chain PC's, interchain interactions are not the dominant forces involved in the stabilization of the lipid bilayer, and as a result there could be behavioral discontinuities between those compounds and their long-chain counterparts, for which the van der Waals interactions would be dominant. Thus, for these short-chain ω-cyclohexyl-PC's, the above considerations and the data presented here suggest that their gel-state conformations may be different from those of their longer chain counterparts and that the dominant forces in the stabilization of their bilayer structures may be those of inter-head-group and head-group–solvent interactions and the steric considerations relating to the accommodation of the size and rigidity of the cyclohexyl end group.

The complexity of the thermotropic phase behavior of these ω-cyclohexyl-PC's makes comparison with other known systems extremely difficult. Although it is clear that these compounds can exhibit gel-state polymorphism that is at least as complex as that of *n*-acyl- or isoacyl-PC's, further comparisons are complicated by a current lack of data concerning the participation, if any, of the cyclohexyl ring in the phenomena observed. Nevertheless, our comparisons with other systems will be based on the assumption that these compounds would have an effective hydrocarbon chain length of $n + 3$, since our studies with molecular models have shown that, in the all-trans conformation, three of the ring carbons would be continuous with the zig-zag line of the linear chain. Given this, the probable "real" chain-melting transition temperatures of the ω-cyclohexyl-PC's (their main cooling exotherms) would appear to be some 15 °C lower than those of comparable *n*-acyl-PC's, and their chain-melting enthalpies (estimated from the high-temperature endotherms of the even-numbered compounds) would appear to be considerably smaller than those of the *n*-acyl-PC's. This may suggest that the gel-state intermediate postulated to be necessary for the formation of the liquid-crystalline state is considerably more loosely packed than the P_{β}' phase of the *n*-acyl-PC's. Similar suggestions have been made for the isoacyl-PC's [see Lewis & McElhaney (1985)]. It is interesting to note, however, that the probable chain-melting temperatures of the ω-cyclohexyl-PC's are in the same range as those of their isoacyl counterparts of comparable effective chain length, though their probable chain-melting enthalpies are considerably lower than those of their comparable isoacyl counterparts. This may suggest that the necessary gel-state intermediate is in a more loosely packed conformation than even that of the isoacyl-PC's. This suggestion is supported by the ^{31}P NMR data, which clearly show that, in cases where the intermediate is expected to be stable (i.e., the gel-state conformations of the even-numbered compounds), the limiting spectral line shape characteristic of fast axially symmetric motion is approached *before* the hydrocarbon chain melts, a condition that would only be feasible in a loosely packed conformation. Another interesting suggestion is the possibility that, in the liquid-crystalline state, the hydrocarbon domain of these ω-cyclohexyl-PC's is more ordered than that of their *n*-acyl-PC's in a comparable state. This seems feasible, since the bulky cyclohexyl end group would probably interfere with the neighboring acyl chains on account of their greater steric and excluded volume effects and the fact that the mobility of the carbons in the center of the lipid bilayer would be severely reduced by being constrained in the cyclohexyl ring. This could be a contributory cause of the relative low chain-melting enthalpy of these compounds and may in part offer an explanation for the fact that, when compared with *n*-acyl-PC's in the liquid-crystalline state, liposomes made from the ω-cyclohexyl-PC's were found to be more resistant to permeation by small molecules (Endo et al., 1982; Benz et al., 1983; Kannenberg et al., 1984).

The characteristic enthalpy changes of three of the compounds described here ($n = 11$–13) are considerably higher than those reported in the literature (Endo et al., 1982; Kannenberg et al., 1984). In addition, the heating endotherms shown here are considerably narrower than those previously reported, even when studied in the low-sensitivity calorimeter. We have observed that, like the methyl iso-branched PC's [see Lewis & McElhaney (1985)], the thermotropic phase behavior of these compounds is extremely sensitive to the presence of impurities, and have correlated the broadening of the heating endotherms with the presence of small-molecule impurities. Thus, we suspect that the differences between the sets of data can be attributed to the higher purity of the samples used in this study.

It is clear from the data presented here that hydrocarbon chain structure can have profound effects on the phase behavior of a lipid bilayer. This implies that the limits within which reasonable extrapolations can be made from the few acyl chain structures that have been thoroughly studied are a lot narrower than has been suspected. In addition, the odd–even discontinuities described here, as well as the anomalous behavior of the short-chain compounds, highlight the pitfalls of using any one member of a series of fatty acids as a representative model of that chain structure. Our results indicate that it may often be necessary to study an entire homologous series of such chain structures to obtain an accurate picture of their behavior in lipid bilayers, especially when the more complex, naturally occurring hydrocarbon-chain structures are studied. Finally, it may be interesting to speculate as to whether the odd–even discontinuities observed here are significant with regard to the biological viability of these fatty acyl chain structures, especially since only the odd-numbered species are naturally occurring. However, we suspect that such is only a reflection of the absence of suitable biosynthetic precursors, since we found that some of the even-numbered fatty acids can support normal growth and function of the *A. laidlawii* B under conditions in which the cell membranes are made homogeneous in such fatty acids (unpublished observations from this laboratory).

ACKNOWLEDGMENTS

We are indebted to Drs. Brian D. Sykes and Manfred Brauer for the availability of time on the NMR spectrometer and for assistance in the acquiring of the NMR spectra and to Charles Seguin for his skillful technical assistance.

Registry No. C_9 PC, 97352-44-8; C_{10} PC, 97352-45-9; C_{11} PC, 77710-69-1; C_{12} PC, 96332-11-5; C_{13} PC, 81326-18-3; C_{14} PC, 97352-46-0; C_{15} PC, 97352-47-1; C_{16} PC, 97352-48-2; C_{17} PC, 97352-49-3; C_{18} PC, 97352-50-6; $\omega_{cy}C_9$, 4352-53-8; $\omega_{cy}C_{10}$, 4355-07-1; $\omega_{cy}C_{11}$, 4277-62-7; $\omega_{cy}C_{12}$, 18017-77-1; $\omega_{cy}C_{13}$, 20913-08-0; $\omega_{cy}C_{14}$, 19708-94-2; $\omega_{cy}C_{15}$, 19708-95-3; $\omega_{cy}C_{16}$, 19708-96-4; $\omega_{cy}C_{17}$, 19708-97-5; $\omega_{cy}C_{18}$, 19708-98-6; adipic acid monomethyl ester, 627-91-8; 1-bromo-3-cyclohexylpropane, 34094-21-8; azelaic acid monomethyl ester, 2104-19-0; cyclohexylmethyl bromide, 2550-36-9; 1,10-decanedioic acid, 111-20-6; 1-bromo-2-cyclohexylethane, 1647-26-3; 1,12-dodecanedioic acid, 693-23-2; 1,14-tetradecanedioic acid, 821-38-5; 1,13-tridecanedioic acid, 505-52-2; 1-bromo-4-cyclohexylbutane, 60439-16-9; cyclohexanebutyric acid, 4441-63-8.

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