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Thermotropic Phase Behavior of Model Membranes Composed of Phosphatidylcholines Containing Cis-Monounsaturated Acyl Chain Homologues of Oleic Acid: Differential Scanning Calorimetric and ^{31}P NMR Spectroscopic Studies†

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ABSTRACT: The thermotropic phase behavior of dioleoylphosphatidylcholine and six of its longer chain homologues was studied by differential scanning calorimetry and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Aqueous dispersions of these compounds all exhibit a single endotherm upon heating but upon cooling exhibit at least two exotherms, both of which occur at temperatures lower than those of their heating endotherm. The single transition observed upon heating was shown by ^{31}P NMR spectroscopy to be a net conversion from a condensed, subgel-like phase (L_c phase) to the liquid-crystalline state. Aqueous ethylene glycol dispersions of these compounds also exhibit single endotherms upon heating and cooling exotherms centered at temperatures lower than those of their corresponding heating endotherm. However, the behavior of the aqueous ethylene glycol dispersions differs with respect to their transition temperatures and enthalpies as well as the extent of "undercooling" observed, and there is some evidence of discontinuities in the cooling behavior of the odd- and even-numbered members of the homologous series. Like the aqueous dispersions, ^{31}P NMR spectroscopy also shows that the calorimetric events observed in aqueous ethylene glycol involve net interconversions between an L_c-like phase and the liquid-crystalline state. However, the L_c phase formed in aqueous ethylene glycol dispersions exhibits a considerably broader powder pattern than that observed in water. This, together with the fact that the transition enthalpies of the aqueous ethylene glycol dispersions are considerably higher than those of the aqueous dispersions, indicates that these lipids form more ordered L_c phases in aqueous ethylene glycol. These results demonstrate that although the presence of a cis double bond can perturb the solid-state packing of the acyl chains, its presence does not preclude the formation of highly ordered subgel-like phases in lipid bilayers. In the particular case of these unsaturated phosphatidylcholines, the formation of their subgel phases is more kinetically favorable than is the case with their saturated *n*-acyl counterparts.

Unsaturated fatty acids are common and widespread constituents of many biological membranes. Of these, the monounsaturated fatty acids are found as membrane lipid constituents of many species of eubacteria, while both the mono- and polyunsaturated fatty acids are major constituents of all eucaryotic cell membranes. The biological importance of unsaturated fatty acids is believed to be related to the fact that their melting points are much lower than those of their saturated counterparts, with the result that membrane lipids containing carbon-carbon double bonds tend to have lower gel/liquid-crystalline phase transition temperatures. A number of studies have shown that a predominance of gel-state lipid is not compatible with the maintenance of "normal" membrane function [for reviews, see McElhaney (1982, 1984)], and for most living organisms, the maintenance of a viable liquid-crystalline cell membrane is achieved primarily by the presence of double bonds in the acyl chains of a significant fraction of their membrane lipids. In addition, other studies have sug-

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gested that the maintenance of an "optimal membrane fluidity" is important (if not essential) for the normal functioning of some biomembranes and the regulation of the "degree of unsaturation" of the membrane lipids may be an integral part of the "homeoviscous adaptive responses" by which an optimal membrane fluidity is maintained [for reviews, see McElhaney (1982) and Cossins (1983)].

Apart from its obvious biological importance, the effect of a double bond on the properties of a lipid bilayer is of considerable interest from a purely physical perspective. The most detailed structural studies so far reported have been the single-crystal, X-ray diffraction structures of oleic acid (Abrahamsson & Ryderstedt-Nahringbauer, 1962; Ernst et al., 1979) and of three polyunsaturated fatty acids, viz., linoleic acid, linolenic acid, and arachidonic acid (Ernst et al., 1979). It is clear from these studies that the presence of a cis double bond has a major effect on the solid-state packing of the fatty acid hydrocarbon chains. Most physical studies of cis unsaturated phospholipids published thus far have not been as detailed or as thorough as has been the case for the *n*-acyl model membrane systems (especially the *n*-acylphosphatidylcholines). Calorimetric studies have shown that the presence of a double bond results in a lowering of the gel/liquid-crystalline phase transition temperature (when compared with the *n*-acyl counterparts) as well as a modest reduction in the enthalpy associated with that process [see Silvius (1982) and Small (1986)]. The magnitude of these effects was shown to be dependent upon the position of the double bond in the acyl chain (Barton & Gunstone, 1975) as well as the configuration of the double bond, i.e., whether cis or trans [see Silvius (1982) and Small (1986)]. Similar trends also emerged from studies on a natural biomembrane (*Acholeplasma laidlawii* B plasma membrane) that was enriched in unsaturated fatty acids (Macdonald et al., 1984, 1985a,b). The most detailed structural studies so far attempted have used ^2H nuclear magnetic resonance (NMR)[1] and ^{19}F NMR spectroscopic techniques to study the effect of double bonds on the orientational ordering and dynamics of the acyl chains in both model and natural lipid bilayers [see Seelig and Seelig (1977), Seelig and Waespe-Sarčevič (1978), Rance et al. (1980), Macdonald et al. (1984, 1985a,b), and Perley et al. (1985) and references cited therein]. Such studies have shown that the presence of a carbon–carbon double bond results in a local perturbation of the methylene segments of adjacent acyl chains and that the gel and liquid-crystalline phases of these unsaturated lipids are less ordered and more ordered, respectively, than the corresponding phases of their saturated *n*-acyl counterparts at comparable reduced temperatures. Such differences in organization may account, in part, for the fact that dioleoylphosphatidylcholine model membranes are more accommodating to the presence of small hydrophobic molecules than are bilayers of dimyristoylphosphatidylcholine (Jacobs & White, 1984a,b).

Despite the obvious biological and physical significance of unsaturated glycerolipids, the thermotropic phase behavior of model membranes composed of unsaturated phospho- and glycolipids has not been well characterized. A number of recent studies on phosphatidylcholine bilayers have shown that changes in the length and structure of the fatty acyl hydrocarbon chains can have marked effects on the phase properties of lipid bilayers, particularly in determining the number and types of gel phases formed [see Lewis and McElhaney (1985a,b), Lewis et al. (1987a,b), and Huang and Mason (1986) and references cited therein]. Many of the complex effects observed in these studies could not have been predicted from previous work. Moreover, it is now apparent that one cannot validly use any single number of a homologous series as a model for all other homologues [for an example, see Lewis and McElhaney (1985b)]. Furthermore, those studies also showed that the structure of the acyl chain can have a marked effect on the way in which the bulk solvent phase affects the phase properties of the lipid bilayer. This may be of special relevance to the studies on the "unsaturated" lipids, since many of the compounds studied exhibit their phase transitions at temperatures below 0 °C. Because of this, many workers have used aqueous ethylene glycol as an antifreeze solvent and observed marked differences in the phase properties of those lipids [see Van Echteld et al. (1980), Nicolay et al. (1986), Smaal et al. (1987), and data compiled by Silvius (1982)]. To date, the physical basis of such behavior is not understood. Given the relative shortage of data on unsaturated lipid bilayers, we have begun a thorough characterization of the thermotropic phase behavior of aqueous and aqueous ethylene glycol dispersions of dioleoylphosphatidylcholine and six of its longer chain homologues. The differential scanning calorimetric and ^{31}P NMR spectroscopic data are presented here.

Materials and Methods

The phosphatidylcholines used in this study were synthesized from commercially available highly purified fatty acids (Nu-Chek Prep Inc.) using the acylation procedure of Patel et al. (1979). The lipids were subsequently purified by methods known to produce highly pure samples [see Lewis and McElhaney (1985a)], lyophilized from benzene, and stored under nitrogen at −20 °C. Differential scanning calorimetric measurements were done by using a Microcal MC-1 or a Microcal MC-2 high-sensitivity scanning calorimeter and a Perkin-Elmer DSC-2C calorimeter equipped with a thermal analysis data station. The ^{31}P NMR spectra were recorded on a Nicolet NT-300WB spectrometer (121.47 MHz for ^{31}P) operating in the Fourier-transform mode with quadrature detection. All spectra were obtained with a spectral width of ±31.25 kHz, an acquisition time of 32.77 ms, a recycle delay of 2 s, and a broad-band proton decoupling power (5 W) switched on only during data acquisition so as to avoid sample heating effects. The spectra were recorded by using single-pulse techniques or the phase-cycled Hahn echo described by Rance and Byrd (1983). The single-pulse acquisition technique was used in routine applications since for a given number of transients, the signal to noise ratios of the spectra obtained were considerably higher than those obtained with the Hahn echo. The single-pulse spectra were all recorded with a 20-μs 90° pulse and a 10-μs preacquisition delay, and the data processing to produce the spectra involved a noise reduction function which resulted in a line broadening of 100 Hz. The samples used in these studies were dispersed in excess water or 50% aqueous ethylene glycol (v/v) by vigorous vortexing at temperatures at least 10 °C above their nominal gel/liquid-crystalline phase transition temperatures. Quantification of the samples was achieved by gas chromatographic analysis using appropriate internal standards as has been described previously (Lewis & McElhaney, 1985a).

Results

Differential Scanning Calorimetry. (A) Thermotropic Phase Behavior in Water. DSC thermograms of aqueous dispersions of the seven PCs studied are shown in Figures 1 and 2. Three of these lipids (n = 18, 19, and 20) exhibited

[1] Abbreviations: DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; PC, phosphatidylcholine.

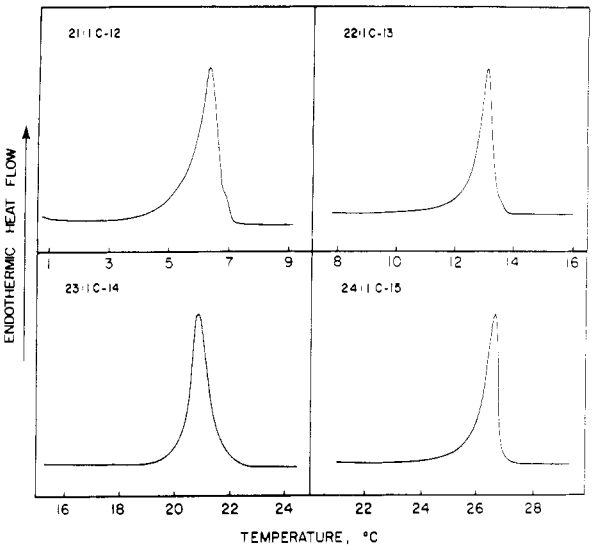

FIGURE 1: High-sensitivity heating endotherms of the cis-monounsaturated acyl-PCs in aqueous dispersion. The thermograms shown were obtained at a scan rate of 10–12 °C h^{-1}.

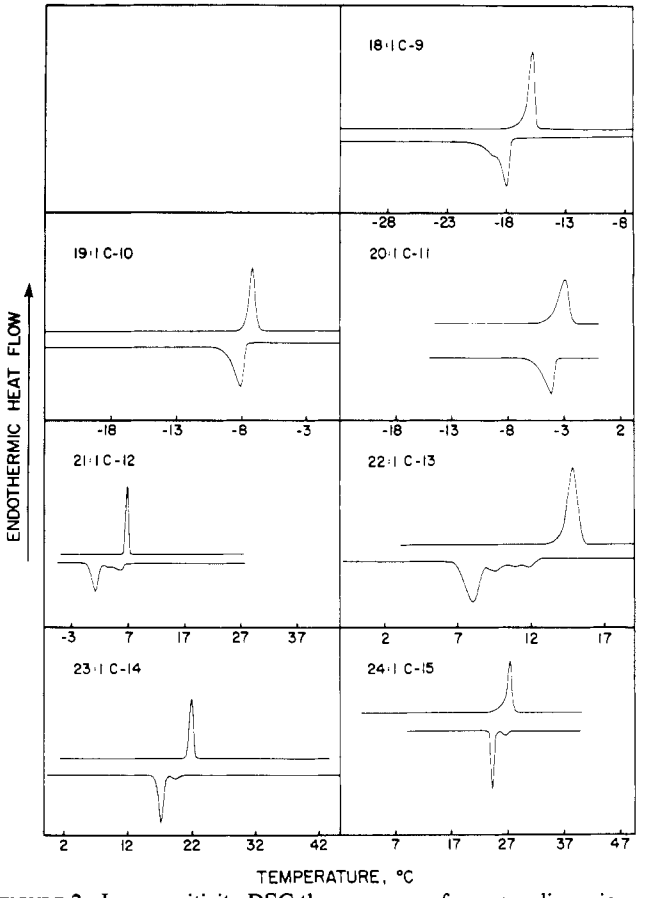

FIGURE 2: Low-sensitivity DSC thermograms of aqueous dispersions of the cis-monounsaturated acyl-PCs. The thermograms shown were recorded at a scan rate of 18.75 °C h^{-1}.

phase transitions below the freezing point of water and were not amenable to high-sensitivity DSC measurements. The heating behavior of all of these lipids is characterized by fairly broad, asymmetric heating endotherms which are "skewed" toward the low-temperature end. These endotherms exhibit a modest heating hysteresis, as evidenced by a slight broadening of the transitions and a small increase in their temperature with increasing scan rate. Once cooled to low temperatures, the observed annealing behavior of these PCs was apparently unaffected by prolonged incubation (8 months) at

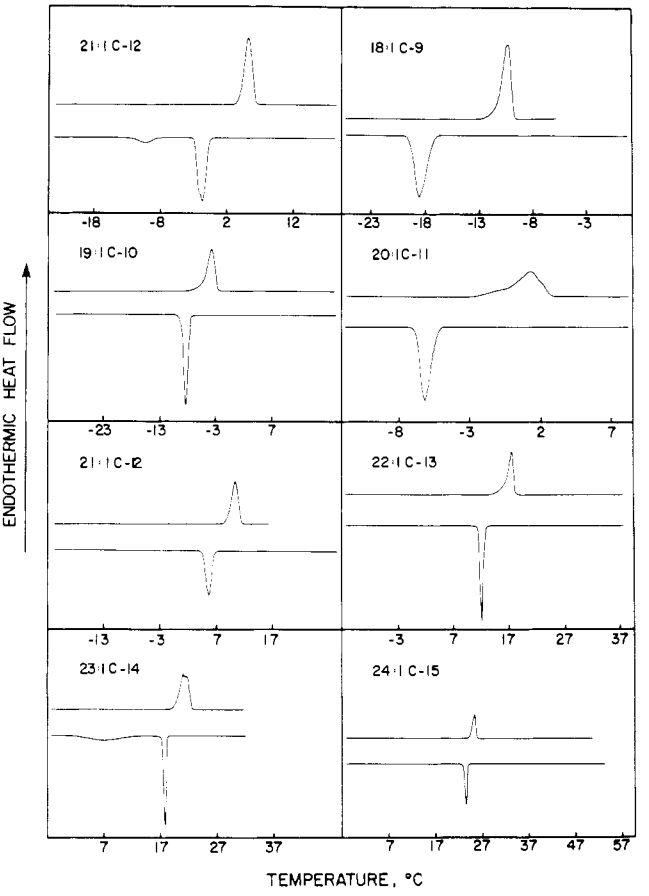

FIGURE 3: Low-sensitivity DSC thermograms of aqueous ethylene glycol dispersions of the cis-monounsaturated acyl-PCs. The thermogram of 21:1c12-PC shown in the left uppermost panel was recorded at a scan rate of 60 °C h^{-1}, and all others were recorded at a scan rate of 18.75 °C h^{-1}.

low temperatures. When examined in the cooling mode, the phase behavior of these PCs is more complex than suggested by their apparently simple heating behavior. The cooling exotherms of all of these lipids are observed at temperatures lower than those of their corresponding heating endotherms, and most of them showed evidence of two cooling exothermic events. The "undercooling" observed in these studies is not the result of classical cooling hysteresis, since their observed behavior is dependent upon the thermal history of the sample and is unaffected by prolonged annealing at temperatures between the heating and cooling peaks. The occurrence of such behavior has been observed in earlier studies on a number of other phosphatidylcholines (Lewis & McElhaney, 1985a,b; Lewis et al., 1987b), and in all cases where it occurs, the single heating endothermic transition was found to be net conversion from a condensed L_c-like phase to the liquid-crystalline state. Our ^{31}P NMR spectroscopic studies have shown that the same is also true of the unsaturated PCs described here.

(B) Thermotropic Phase Behavior in Aqueous Ethylene Glycol. The fact that most unsaturated lipids exhibit phase transitions at temperatures below the freezing point of water has encouraged many workers to include ethylene glycol in the aqueous phase to prevent the freezing of the bulk solvent phase. The DSC thermograms of this series of unsaturated acyl chain PCs dispersed in 50% aqueous ethylene glycol are shown in Figure 3. Their heating endotherms are qualitatively similar to those observed in water, but the transition temperatures and associated enthalpy changes (see Table I) are significantly different. Like the aqueous dispersions, the cooling exotherms of these lipids are observed at temperatures

Table I: Transition Temperatures and Transition Enthalpies of Heating Endothermic Phase Transitions of Cis-Monounsaturated Acyl-PCs

	water		aqueous ethylene glycol	
PC[a]	T_m (°C)	ΔH_{cal}[b]	T_m (°C)	ΔH_{cal}
18:1c9	−17.3	7.8	−11.8	15.6
19:1c10	−8.6	9.9	−5.2	16.5
20:1c11	−4.3	11.4	−0.1	15.0
21:1c12	6.5	11.1	9.1	25.5
22:1c13	13.2	15.1	16.0	22.5
23:1c14	20.9	13.7	19.7	22.4
24:1c15	26.7	16.5	24.0	19.2

[a] The shorthand notation used to describe these lipids is *N:icx* where *N* is the number of carbon atoms per acyl chain, *i* is the number of carbon–carbon double bonds, *c* indicates a cis double bond configuration, and *x* is the position of the double-bonded carbon nearest to the carboxyl carbon. [b] Values quoted in kilocalories per mole.

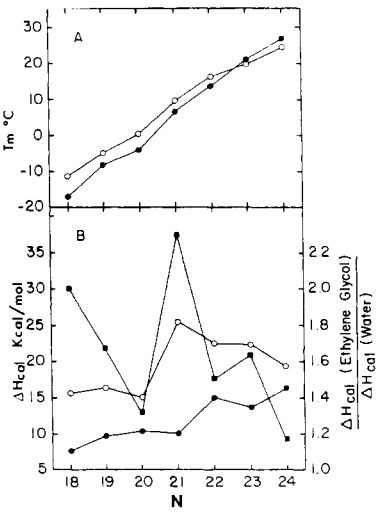

FIGURE 4: Chain length dependence of the calorimetric parameters measured for the cis-monounsaturated acyl-PCs. (A) Transition temperatures for dispersions in water (●) and dispersions in aqueous ethylene glycol (○). (B) Transition enthalpies for dispersion in water (●) and dispersion in aqueous ethylene glycol (○). Ratio of transition enthalpy of aqueous ethylene glycol dispersion to transition enthalpy of aqueous dispersion (■).

below those of the corresponding heating endotherms, and the event(s) observed usually occur(s) at temperatures lower than those observed in water. The cooling behavior of the aqueous ethylene glycol dispersions also shows evidence of discontinuities in the behavior of the odd- and even-numbered members of this series of lipids. The even-numbered members exhibit single cooling exotherms of enthalpy comparable to that of the heating endotherms, whereas, depending upon the cooling rate, their odd-numbered counterparts can exhibit two exotherms of which the higher temperature event usually contributed the major fraction of the total enthalpy. Although the total enthalpy change measured was comparable to that observed on heating, the distribution of the total enthalpy between the two exotherms is markedly dependent upon the cooling rate, as is vividly demonstrated by the two cooling exotherms of di-*cis*-(12,13)-heneicosenoyl-PC shown in Figure 3 and the data listed in Table II. These data indicate that the odd-numbered members of this homologous series of PCs can, at fast scan rates, exhibit two cooling exotherms and that there is a significant kinetic component to the behavior of these odd-numbered PCs when dispersed in aqueous ethylene glycol.

(*C*) *Calorimetric Data.* The heating endothermic transition temperatures both of aqueous and of aqueous ethylene glycol dispersions of these monounsaturated acyl-PCs are listed in Table I, along with the enthalpy changes associated with these processes. In both solvent phases, these lipids exhibit the expected increase in transition temperatures as the acyl chain lengthens. The observed phase transition temperatures are not a smooth and regular function of acyl chain length (see Figure 4A), and deviations of 2–5 °C from either a linear or a curved function are typical. These deviations, which are at least 25 times greater than the precision to which the temperatures were measured, exhibit a consistent tendency toward odd–even discontinuities in the transition temperatures, especially in the case of the shorter chain lipids ($n \leq 22$). Since the transition temperatures of the "normal" gel/liquid-crystalline phase transition (i.e., processes analogous to the $P_{\beta'} \rightarrow L_\alpha$ transition of the *n*-acyl-PCs) of lipid bilayers can be described by a smooth function of acyl chain length [see Lewis et al. (1987) and references cited therein], it is clear there must be other events (probably solid-phase processes) which are concomitant with the expected normal chain-melting process. The data listed in Table I also show that the transition enthalpies measured in aqueous ethylene glycol dispersions are considerably higher than those measured in water, and we have correlated this increase in transition enthalpy with the formation of a more ordered L_c phase by ^{31}P NMR spectroscopy (see below). However, as shown in Figure 4B, the effect of aqueous ethylene glycol on the measured enthalpy change is not uniform across the homologous series. Figure 4B clearly shows a marked discontinuity between the three shorter chain PCs studied and their four longer chain homologues as regards the effect of ethylene glycol on the measured transition enthalpy. Nevertheless, both groups show an overall (though not continuous) trend toward a decrease in the magnitude of the effect of aqueous glycol with increasing acyl chain length.

Although the heating endotherms and cooling exotherms of these lipids are observed at different temperatures, the measured total enthalpy changes are similar, provided that the scan rates (especially cooling rates) are sufficiently slow (<60 °C h^{-1}). However, the cooling rate does affect the distribution of the total enthalpy between the various exothermic events observed when the odd-numbered PCs are dispersed in aqueous ethylene glycol. A summary of the scan rate effect is shown in Table II, and from the data shown therein, it is clear that the appearance of the two exotherms is most likely a kinetic phenomenon, since the fraction of the total enthalpy change contributed by the "low-temperature" exothermic component decreases with decreasing cooling rate. The data also show

Table II: Effect of Cooling Rate on the Transition Temperatures and Distribution of Enthalpy between Cooling Exothermic Transitions Observed in Aqueous Ethylene Glycol Dispersions of Odd-Numbered Cis-Monounsaturated Acyl-PCs

	cooling rate = 18.75 °C h^{-1}				cooling rate = 60 °C h^{-1}			
	high-temp exotherm		low-temp exotherm		high-temp exotherm		low-temp exotherm	
PC	T_m (°C)	% total enthalpy	T_m (°C)	% total enthalpy	T_m (°C)	% total enthalpy[a]	T_m (°C)	% total enthalpy[a]
19:1c10	−9.8	100			−12.5	94.2	−27.4	5.8
21:1c12	4.4	100			1.9	92.6	−6.9	7.4
23:1c14	16.5	84	5.6	16	16	71	1.6	29

[a] The sum of the total enthalpy change measured is listed in Table I.

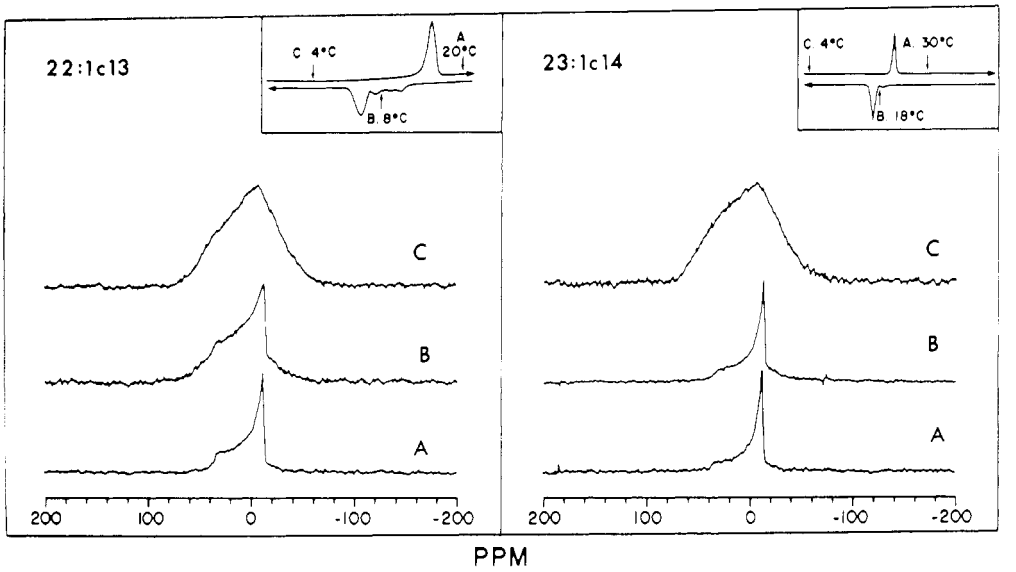

FIGURE 5: Proton-decoupled ^{31}P NMR spectra of representative cis-monounsaturated acyl-PCs. The spectra were acquired by using the single-pulse acquisition procedure, and the insets show the DSC thermograms of the lipids with the arrows indicating the temperatures and thermal histories of the samples used.

that the above trend gets less pronounced as the acyl chain length increases and suggest that the kinetics of the process(es) involved get slower as the acyl chain lengthens.

(*D*) *^{31}P Nuclear Magnetic Resonance Spectroscopy.* Illustrated in Figure 5 are the ^{31}P NMR spectra of aqueous dispersions of representative odd- and even-numbered members of this homologous series of PCs. The spectra were acquired by using the single-pulse acquisition procedure at temperatures which bracket the main thermotropic events observed by DSC. At temperatures well above that of the heating endothermic transition (spectra A), the lipids exhibit powder patterns indicative of the fast axially symmetric motion of the phosphate head group in a phospholipid bilayer [see Seelig (1978) and Campbell et al. (1979)]. When cooled to temperatures well below those of the thermotropic events seen by DSC (spectra C), the lipids exhibit a broad powder pattern (basal line width $\simeq$ 120 ppm) indicative of slow axially asymmetric motion of the phosphate head group on the ^{31}P NMR time scale (Seelig, 1978; Campbell et al., 1979). Such powder patterns have been exhibited by a number of other PCs [see Fuldner (1981), Lewis et al. (1984, 1987a), Mantsch et al. (1985), and Lewis and McElhaney (1985b)] which form condensed, subgel-like phases (L_c phases). It is thus clear that the single endotherm observed upon heating these lipids involves a net conversion from a L_c-like phase to the liquid-crystalline state (L_α phase).

Spectra B shown in Figure 5 illustrate the type of powder patterns that are obtained when the samples are cooled from their respective L_α phases to temperatures which bracket the two cooling exotherms observed by DSC. The spectra shown appear to be a composite of axially symmetric powder patterns similar to that characteristic of their respective L_α phases and a broad component of basal line width near 90 ppm. Similar powder patterns have been observed in some of the gel states of the *dl*-anteisoacyl-PCs (Lewis et al., 1987b), in which case the data were interpreted in terms of the coexistence of phases in which the phosphate head group had different motional characteristics. To investigate this possibility, we have exploited the expected difference in the transverse relaxation rates of the "broad" and "narrower" components of the spectrum by the use of the Hahn echo pulse sequence, which, by a judicious choice of delay times, can selectively discriminate against the broad component of the spectrum [see Rance and Byrd (1983)]. The spectra shown in Figure 6 were obtained

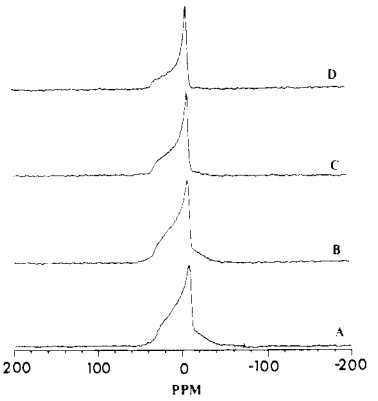

FIGURE 6: Proton-decoupled ^{31}P NMR spectra of di-*cis*-13,14-docosenyl-PC. The sample used was cooled to 8 °C from room temperature, and the spectra were acquired by using the phase-cycled Hahn echo. The spectra shown were acquired with the delay between the $\pi/2$ and π pulses set at (A) 60, (B) 100, (C) 300, and (D) 600 μs.

by using Hahn echo pulse sequences in which the delay between the $\pi/2$ and π pulses was varied. The contribution of the broad component to the spectrum clearly decreases as the interpulse delay increases. At sufficiently long delay times ($\geq$600 μs), its contribution to the observed spectrum is negligible, so that the spectrum adopts the axially symmetric powder pattern normally expected of the L_α phase of phospholipid bilayers. It is thus clear that spectra B shown in Figure 5 are a composite of two components with different transverse relaxation times. The broader component appears to be similar in its ^{31}P NMR characteristics to the $L_{\beta'}$ gel state of saturated straight-chain acyl PCs, judging from its basal line width ($\simeq$90 ppm) and our estimates of its transverse relaxation time ($\simeq$250–300 μs), while the narrower component exhibits the ^{31}P NMR characteristics indicative of fast axially symmetric motion of a phospholipid molecule in a bilayer conformation. The persistence of the latter thus suggests that for a large fraction of the population of PC molecules, the structural changes coincident with the higher temperature exothermic event do not impose any significant restrictions on the mobility of the phosphate head group. This could be the result of the coexistence of gel and liquid-crystalline lipid at those temperatures or of the formation of a very loosely packed

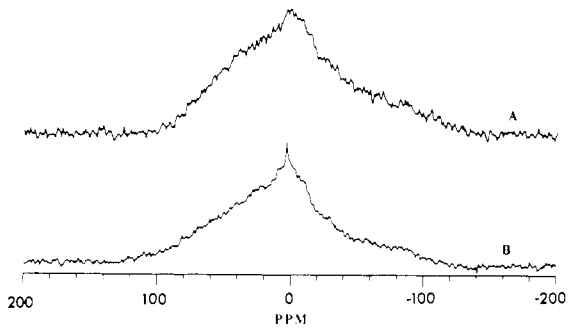

FIGURE 7: Proton-decoupled ^{31}P NMR spectra of aqueous ethylene glycol dispersions of (A) di-*cis*-13,14-docosenyl-PC and (B) di-*cis*-14,15-tricosenoyl-PC at 2 °C. The spectra were acquired by using the single-pulse procedure. The spike in spectrum B at 5 ppm downfield arises from phase-related noise and is not attributable to the presence of any isotropic or liquid-crystalline components in the sample. If present, such components would have produced sharp and intense signals at 0 and 13 ppm upfield, respectively.

gel state in which the motion of the phosphate head group is virtually indistinguishable (by ^{31}P NMR spectroscopy) from that of the liquid-crystalline state. The existence of such a gel state has been reported for the anteisoacyl-PCs (Lewis et al., 1987a) and for the even-numbered ω-cyclohexyl-PCs (Lewis & McElhaney, 1985b).

We also used ^{31}P NMR spectroscopy to determine whether the obvious differences in the thermotropic properties of the aqueous and aqueous ethylene glycol dispersions of these lipids were reflected in the motional properties of their phosphate head groups. Illustrated in Figure 7 are the ^{31}P NMR spectra of aqueous ethylene glycol dispersions of the representative PCs used for ^{31}P NMR studies (22:1c13-PC and 23:1c14-PC) at temperatures well below the range of their heating endotherms or cooling exotherms (4 °C). It is immediately apparent that these spectra differ significantly from those of the aqueous dispersions under the same temperature conditions (compare with spectra C in Figure 5). The powder patterns shown in Figure 7 are considerably broader (basal line width $\simeq$180–200 ppm) and begin to assume some of the features of the so-called rigid-limit powder pattern usually observed with solid phospholipid powders. It is thus clear that when dispersed in aqueous ethylene glycol, these phospholipids form more ordered L_c phases than those usually formed in aqueous dispersions. These ^{31}P NMR spectroscopic observations indicate that, in the L_c phases formed when dispersed in aqueous ethylene glycol, the phosphate head groups are not completely immobilized on the ^{31}P NMR time scale though their motions are considerably more restricted than when dispersed in water under the same experimental conditions.

Discussion

It is not unexpected that the calorimetrically determined gel/liquid-crystalline phase transition temperatures of these cis-monounsaturated acyl-PCs are considerably lower than those of their saturated counterparts. However, the fact that they form ordered L_c-like gel phases and that they appear to form such phases more rapidly than their saturated counterparts is somewhat surprising, given that a cis carbon–carbon double bond would be expected to perturb the solid-state packing of the acyl chains [see Abrahamsson and Ryderstedt-Nahringbauer (1962) and Ernst et al. (1979)]. Several recent studies have shown that the formation of condensed, L_c-like phases in PC bilayers is promoted by the presence of substitutions near the methyl terminus of the hydrocarbon chain (Lewis & McElhaney, 1985a,b; Lewis et al., 1987a), by a reduction in chain length (Lewis & McElhaney, 1985a,b; Lewis et al., 1987a,b), or by a marked difference in chain lengths [see Huang and Mason (1986) for a review]. On the basis of the above observations, the marked tendency of these unsaturated lipids (which have equally long hydrocarbon chains and no large group substituents near their methyl termini) to form L_c phases is not easily understood. However, if the hydrocarbon chains on these PCs are viewed as shorter chain alkanoic acids (8–14 carbon atoms) which have 1,2-*cis*-decenyl groups substituted to their ω-carbon atoms, the relative ease with which these PCs form L_c-like phases can be easily accommodated within the framework described above, since those lipids would be of short to medium acyl chain length and have a large and potentially disruptive group substituted to the ω-carbon position. Such a view of these acyl chain structures may not be too extreme when examined against the background of the single-crystal X-ray data published so far (Abrahamsson & Ryderstedt-Nahringbauer, 1962; Ernst et al., 1979). Those studies have shown that in the solid state, the cis carbon–carbon double bond induces a marked departure in the alignment of the distal methylene segments from the "zig-zag line" of the methylene segments near the carboxyl group. In fact, the "packing requirements" of the cis carbon–carbon double bond, and the methylene segments distal to it, are so different from those of the other methylene groups that a description of the system as a large "disruptive" 1,2-*cis*-decenyl group substituted to the ω-carbon of an *n*-acyl chain may not be a bad approximation.

The fact that the endothermic transitions seen in the heating thermograms of these PCs are $L_c \rightarrow L_\alpha$ transitions has some implications with respect to the way in which the calorimetric data reported here, and in the literature, should be interpreted. We suspect that the normal acyl chain-melting process (i.e., the processes analogous to the $P_{\beta'} \rightarrow L_\alpha$ transition of the *n*-acyl-PCs) probably contributes only a small fraction of the total calorimetric enthalpy change observed. This conclusion is supported by the observation that upon cooling an aqueous dispersion, the higher temperature exothermic event, which may be analogous to the conversion of the L_α phase to a $P_{\beta'}$-like or $L_{\beta'}$-like phase, usually contributes less than 30% of the total enthalpy change. Hence, our calorimetric data would suggest that the "normal" chain-melting process may contribute a maximum of 30% of the total enthalpy change measured. Thus, the cis carbon–carbon double bond is probably a lot more disordering in its effect on the energetics of the chain-melting process of lipids than has been assumed previously. In particular, these studies may offer a rationale for the fact that Barton and Gunstone (1975) observed that the presence of a cis double bond in an acyl chain results in a substantial decrease in the gel/liquid-crystalline phase transition temperature (particularly when present near the middle of the acyl chain) with only a relatively small decrease in the transition enthalpy. We suspect that for most of the unsaturated PCs studied by Barton and Gunstone (1975), the normal chain-melting process probably contributed only a small fraction of the total enthalpy change observed and that most of the enthalpy change originated from gel-phase interconversions involving their respective L_c phases.

The nature of the L_c phase formed by these PCs is clearly affected by the composition of the bulk aqueous phase. This should not be surprising since changes in the hydration levels at the hydrophobic/hydrophilic interfacial region of lipid bilayers are believed to be one of the processes occurring during interconversions involving L_c phases (Cameron & Mantsch, 1982). The addition of significant quantities of solutes like ethylene glycol to the bulk aqueous phase will certainly de-

crease the chemical activity of water, and we suspect that the sensitivity of these and other lipids to the presence of ethylene glycol is largely due to such effects. Indeed, effects related to marked changes in the chemical activity of water may also offer a rationale for the discontinuity in the data shown in Figure 4B, since an obvious difference between the shorter (n = 18–20) and longer chain lipids is the fact that for aqueous dispersions of the shorter chain PCs studied, the bulk solvent phase was frozen at all temperatures at which calorimetric measurements were made. These unsaturated PCs also appear to be more sensitive to the changes in the bulk solvent phase than a number of other PCs studied [see Lewis and McElhaney (1985a,b) and Lewis et al. (1987a)], and the reasons why this should be the case are not immediately obvious. We have also shown here that a change from an aqueous to an aqueous ethylene glycol solvent phase results in the formation of a more highly ordered L_c phase and that the formation of such a structure correlates well with the higher transition enthalpy observed in that solvent (this work; Van Echteld et al., 1980). A previously published study (Smaal et al., 1987) has also suggested that such a change in the bulk solvent phase may affect the acyl chain order in the liquid-crystalline state of these monounsaturated acyl-PCs.

Our results also suggest that there may be a significant kinetic component to the calorimetric behavior observed with these unsaturated PCs, and this is generally consistent with the expectation that the formation of L_c phases is a relatively slow process [see Chen et al. (1980) and Lewis et al. (1984)]. The formation of the L_c phases of these unsaturated PCs is evidently faster than that of their saturated counterparts, and it is also apparent that the kinetics of the observed phase transitions of the odd- and even-numbered members of this homologous series of lipids differ markedly and become slower with increasing acyl chain length, as was also observed with branched and linear saturated PC bilayers [see Lewis and McElhaney (1985a), Yang et al. (1986), and Lewis et al. (1987a,b)]. However, these kinetic considerations also raise some issues pertinent to the structure and stability of the L_c phases formed. First, the odd–even discontinuities in the kinetics of the transformations involving the L_c phases formed, along with the other odd–even discontinuities observed, are probably indicative of a gel-state structure in which the acyl chains are tilted to the bilayer normal [for a discussion of the physical basis of odd–even discontinuities in the behavior of long-chain hydrocarbon compounds, see Broadhurst (1962)]. Indeed, the single-crystal X-ray studies published so far suggest that a strong tilting of the acyl chains may be necessary to accommodate the "solid-state packing requirements" of the cis double bond and the methylene segments distal to it (Abrahamsson & Ryderstedt-Nahringbauer, 1962; Ernst et al., 1979). Second, the kinetic considerations raised may be relevant to a consideration of the thermodynamic stability of the L_c-like gel states of these lipids, especially those formed in water. This has assumed added significance in light of a number of recent studies which have shown that the formation of apparently stable L_c phases of many PCs is a complex, multistage process, which in some cases may occur over a time scale of several months or even years (Lewis et al., 1984, 1987b; Finegold & Singer, 1984; Silvius et al., 1985; Tristram-Nagle et al., 1987). Since it is clear that the unsaturated lipids described here can form more stable L_c phases in aqueous ethylene glycol than in water, it is logical to suggest that the L_c-like gel phases formed in aqueous dispersions are metastable states. However, we have not yet observed any tendency for these L_c phases to convert to more stable structures, even after prolonged incubation (up to 8 months) at low temperatures or exposure to various regimes of low-temperature incubation. Still, we cannot rule out the possibility that in aqueous dispersions, there may be a thermodynamic barrier against the formation of more ordered structures than those observed so far.

It is clear from this investigation, and from a number of studies on other homologous series of PCs [see Lewis and McElhaney (1985a,b), Yang et al. (1986), Lewis et al. (1987a,b), and Mantsch et al. (1985, 1987)], that the length and structure of an acyl chain as well as the number of carbon atoms present (i.e., whether odd or even) can affect the phase behavior of a lipid bilayer in complex and subtle ways which cannot be readily predicted. The unexpectedly complex phase behavior of these unsaturated PCs effectively underscores the above and also highlights the need for more studies aimed at a thorough characterization of the effect of a double bond on the structure of lipid bilayers.

Registry No. 18:1c9-PC, 10015-85-7; 19:1c10-PC, 112087-40-8; 20:1c11-PC, 104757-69-9; 21:1c12-PC, 112087-41-9; 22:1c13-PC, 56649-39-9; 23:1c14-PC, 112087-42-0; 24:1c15-PC, 86288-10-0; ethylene glycol, 107-21-1.

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Evidence for Targeted Gene Delivery to Hep G2 Hepatoma Cells in Vitro†

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ABSTRACT: We have developed a system for targeting foreign DNA to hepatocytes in vitro using a soluble DNA carrier that takes advantage of receptor-mediated endocytosis to achieve internalization. The idea is based on the fact that hepatocytes possess a unique receptor that binds and internalizes galactose-terminal (asialo)glycoproteins. To create a targetable carrier system that could bind DNA in a nondeforming manner, we used poly(L-lysine) to bind DNA in a strong but noncovalent interaction. An asialoglycoprotein, asialoorosomucoid (AsOR), was chemically coupled to poly(L-lysine) to form an asialoorosomucoid–poly(L-lysine) conjugate. Various proportions of conjugate to DNA were tested to determine conditions that maximized DNA content in a soluble complex and that limited solubility of complexes. To test the targetable gene delivery system, AsOR–poly(L-lysine) conjugate was complexed to the plasmid pSV2 CAT containing the gene for chloramphenicol acetyltransferase (CAT) driven by an SV-40 promoter. We tested this complex using a model system consisting of human hepatoma cell line Hep G2 [asialoglycoprotein receptor (+)], hepatoma SK-Hep 1, IMR-90 fibroblasts, and uterine smooth muscle [receptor (−)] cells. Each cell line was incubated with 0.2 μm filtered AsOR–poly(L-lysine)–DNA complex or controls consisting of DNA plus AsOR, DNA plus poly(L-lysine), or DNA alone. Cells were assayed for the presence of CAT activity as a measure of gene transformation. SK-Hep 1, IMR-90, and smooth muscle [receptor (−)] cells produced no detectable acetylated chloramphenicol derivatives under any of these conditions. However, Hep G2 [receptor (+)] cells incubated with the AsOR–poly(L-lysine)–DNA complex *were* transformed as indicated by the appearance of CAT activity (0.028 CAT unit/10^6 cells).

Foreign genes have been introduced into mammalian cells in vitro by a variety of methods in order to study gene regulation. The most popular technique employs a precipitation method in which DNA is coprecipitated with calcium phosphate to form insoluble particles (Graham & Van der Eb, 1973). A proportion of these precipitates becomes internalized within host cells by phagocytosis (Loyter et al., 1982). Following internalization, some of the DNA avoids degradation and eventually enters the nucleus, resulting in expression of new genes (Graham & Van der Eb, 1973). We wondered whether DNA, in the proper form, could similarly survive a receptor-mediated internalization. Our objective was to develop a simple, soluble DNA carrier system to target DNA specifically to hepatocytes using receptor-mediated endocytosis. The idea is based on the following concepts: (1) Hepatocytes possess *unique* receptors that bind and internalize galactose-terminal (asialo)glycoproteins (Ashwell & Morell, 1974). Coupling of DNA to an asialoglycoprotein could permit internalization of DNA via asialoglycoprotein receptors. However, chemical coupling of DNA to a carrier could alter the DNA and prevent proper expression of the genes. To circumvent this problem, we took advantage of the fact that (2) DNA can bind polycations, e.g., poly(L-lysine), in a strong, noncovalent interaction forming *soluble* complexes (Li et al., 1973). (3) Lastly, chloramphenicol acetyltransferase (CAT)[1]

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