Thermotropic Properties of Synthetic Chain-Substituted Phosphatidylcholines: Effect of Substituent Size, Polarity, Number, and Location on Molecular Packing in Bilayers

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Abstract: Differential scanning calorimetry has been applied to aqueous suspensions comprised of synthetic phospholipids on whose chains reside substituents of diverse size (methyl, *n*-butyl, phenyl), polarity (alkyl, keto), number (one or both chains), and location (carbons 4, 6, 8, 10, 12, or 16 of 18-carbon chains). Attention focused primarily on the "main" phase transition in the lipid bilayers resulting from disordering ("melting") of the hydrocarbon tails. The thermotropic behavior revealed, among the more interesting observations, that alkyl groups at the center of the chains are far more disruptive than groups at either end. For example, distearoylphosphatidylcholine (DSPC) has a main transition temperature (T_c) of 54.8 °C. Methyl substitution at carbons 4, 10, and 16 of the second chain gives T_c values of 41.5, 5.6, and 38.5 °C, respectively. Similarly, DSPC has a transition enthalpy, ΔH_{cal}, of 10.6 kcal/mol. Methyl substitution at carbons 4, 10, and 16 of the second chain gives ΔH_{cal} values of 8.8, 3.7, and 9.8 kcal/mol, respectively. This remarkable midchain effect is rationalized on a molecular basis and reconciled with earlier work by others showing that mobility is restricted in the first half of the chain after which motional freedom and disorder increase uniformly up to the terminal methyl. Systematic "structure–activity" studies of lipids, such as presented here, are important in testing membrane models and in controlling membrane properties through rational design of the synthetic lipids.

In 1884 it was written that phospholipids are the "centre, life, and chemical soul of all bioplasm". "Amongst [their] properties none are more deserving of further inquiry than those which may be described as their power of colloidation".[1] In modern terms, "colloidation" refers to the molecular bilayers that phospholipids spontaneously form when mixed with water.[2-8] These bilayers undergo thermal transitions[2,9-12] owing to changes in molecular packing within the hydrocarbon and head-group regions. Studies of such transitions[11-20] have been important in developing models of biological membranes.[5] The present work deals with the thermotropic behavior of synthetic phospholipids bearing diverse substituents on their hydrocarbon chains. Although it has already been noted that chain substitution leads to altered thermal properties,[21-23] we have, for the first time, systematically varied the size, polarity, number, and location of the perturbing groups. Our goal is to elaborate the principles governing chain packing and, ultimately, to control membrane permeability via the rational design and synthesis of lipid molecules.

Curiously, several forms of life, e.g., certain genera of eubacteria,[24,25] possess branched-chain phospholipids as membrane constituents. Thus, fatty acid chains with methyl,[26] cyclopropyl,[27-29] and cyclohexyl[30] branches have been identified in nature. Perhaps branching serves to modify structural and functional parameters as do olefinic sites in mammalian membranes.[31,32] Calorimetric studies on phosphatidylcholines substituted with alkyl groups near the chain termini[26,30,33-40] seem to bear this out.

Differential scanning calorimetry (DSC),[41,42] our primary investigative tool, provides a powerful means for detecting changes in lipid packing without the need of exogenous probes (that can themselves perturb the bilayer). DSC monitors the thermally induced transition of phospholipid bilayers from a relatively ordered state (below the transition temperature, T_c) to a more disordered, fluidlike state (above the T_c). Tracings of excess heat capacity vs temperature provide (a) T_c, the temperature at the maximum in the excess heat capacity profile, (b) ΔH_{cal}, the total enthalpy associated with the transition, (c) ΔH_{vH}, the van't Hoff enthalpy, and (d) $\Delta H_{vH}/\Delta H_{cal}$ = CU, the cooperativity unit. Below T_c, the "gel" phase has its hydrocarbon chains existing mainly in all-trans configurations. Both chain motion and the number of gauche linkages increase in the "liquid-crystal" phase above T_c.[43,44] DSC-derived parameters of our synthetic lipids permitted us to study the effect of branching on the main gel-

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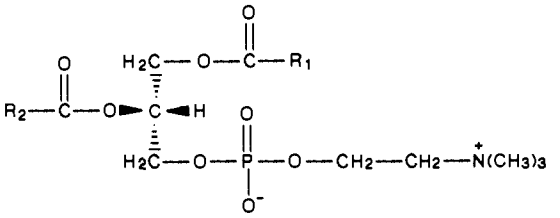
Table I. Structures of Synthetic Phosphatidylcholines



compd	R_1	R_2	compd	R_1	R_2
DSPC	$(CH_2)_{16}CH_3$	$(CH_2)_{16}CH_3$	(1,2)-PC-4M	$(CH_2)_2CH(CH_3)(CH_2)_{13}CH_3$	$(CH_2)_2CH(CH_3)(CH_2)_{13}CH_3$
(2)-PC-4M	$(CH_2)_{16}CH_3$	$(CH_2)_2CH(CH_3)(CH_2)_{13}CH_3$	(1,2)-PC-6M	$(CH_2)_4CH(CH_3)(CH_2)_{11}CH_3$	$(CH_2)_4CH(CH_3)(CH_2)_{11}CH_3$
(2)-PC-6M	$(CH_2)_{16}CH_3$	$(CH_2)_4CH(CH_3)(CH_2)_{11}CH_3$	(1,2)-PC-8M	$(CH_2)_6CH(CH_3)(CH_2)_9CH_3$	$(CH_2)_6CH(CH_3)(CH_2)_9CH_3$
(2)-PC-8M	$(CH_2)_{16}CH_3$	$(CH_2)_6CH(CH_3)(CH_2)_9CH_3$	(1,2)-PC-10M	$(CH_2)_8CH(CH_3)(CH_2)_7CH_3$	$(CH_2)_8CH(CH_3)(CH_2)_7CH_3$
(2)-PC-10M	$(CH_2)_{16}CH_3$	$(CH_2)_8CH(CH_3)(CH_2)_7CH_3$	(1,2)-PC-12M	$(CH_2)_{10}CH(CH_3)(CH_2)_5CH_3$	$(CH_2)_{10}CH(CH_3)(CH_2)_5CH_3$
(2)-PC-12M	$(CH_2)_{16}CH_3$	$(CH_2)_{10}CH(CH_3)(CH_3)_5CH_3$	(1,2)-PC-16M	$(CH_2)_{14}CH(CH_3)CH_2CH_3$	$(CH_2)_{14}CH(CH_3)CH_2CH_3$
(2)-PC-16M	$(CH_2)_{16}CH_3$	$(CH_2)_{14}CH(CH_3)CH_2CH_3$	(1,2)-PC-8P	$(CH_2)_6CH(Ph)(CH_2)_9CH_3$	$(CH_2)_6CH(Ph)(CH_2)_9CH_3$
(2)-PC-8P	$(CH_2)_{16}CH_3$	$(CH_2)_6CH(Ph)(CH_2)_9CH_3$	(1,2)-PC-12P	$(CH_2)_{10}CH(Ph)(CH_2)_5CH_3$	$(CH_2)_{10}CH(Ph)(CH_2)_5CH_3$
(2)-PC-12P	$(CH_2)_{16}CH_3$	$(CH_2)_{10}CH(Ph)(CH_2)_5CH_3$	(1,2)-PC-4B	$(CH_2)_2CH(n\text{-}Bu)(CH_2)_{13}CH_3$	$(CH_2)_2CH(n\text{-}Bu)(CH_2)_{13}CH_3$
(2)-PC-12B	$(CH_2)_{16}CH_3$	$(CH_2)_{10}CH(n\text{-}Bu)(CH_2)_5CH_3$	(1,2)-PC-8B	$(CH_2)_6CH(n\text{-}Bu)(CH_2)_9CH_3$	$(CH_2)_6CH(n\text{-}Bu)(CH_2)_9CH_3$
(2)-PC-8K	$(CH_2)_{16}CH_3$	$(CH_2)_6C(=O)(CH_2)_9CH_3$	(1,2)-PC-10B	$(CH_2)_8CH(n\text{-}Bu)(CH_2)_7CH_3$	$(CH_2)_8CH(n\text{-}Bu)(CH_2)_7CH_3$
			(1,2)-PC-12B	$(CH_2)_{10}CH(n\text{-}Bu)(CH_2)_5CH_3$	$(CH_2)_{10}CH(n\text{-}Bu)(CH_2)_5CH_3$
			(1,2)-PC-4K	$(CH_2)_2C(=O)(CH_2)_{13}CH_3$	$(CH_2)_2C(=O)(CH_2)_{13}CH_3$
			(1,2)-PC-6K	$(CH_2)_4C(=O)(CH_2)_{11}CH_3$	$(CH_2)_4C(=O)(CH_2)_{11}CH_3$
			(1,2)-PC-8K	$(CH_2)_6C(=O)(CH_2)_9CH_3$	$(CH_2)_6C(=O)(CH_2)_9CH_3$
			(1,2)-PC-10K	$(CH_2)_8C(=O)(CH_2)_7CH_3$	$(CH_2)_8C(=O)(CH_2)_7CH_3$
			(1,2)-PC-12K	$(CH_2)_{10}C(=O)(CH_2)_5CH_3$	$(CH_2)_{10}C(=O)(CH_2)_5CH_3$

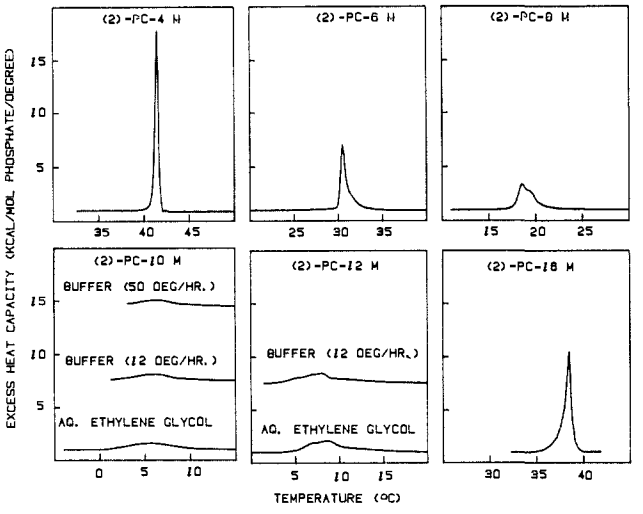
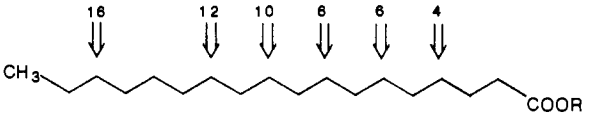


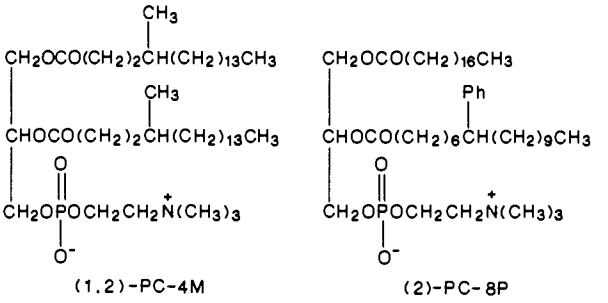
Figure 1. DSC thermograms for six phosphatidylcholines substituted on the second 18-carbon chain with a methyl group at positions 4, 6, 8, 10, 12, or 16. Unless otherwise indicated, suspensions were run in aqueous buffer solution at a scan rate of 50 °C/h.

to-liquid crystal transition and, by inference, on the molecular packing in the gel and liquid-crystal forms of the bilayers.

Phosphatidylcholines have been synthesized,[45] in an extremely high state of purity, with substituents (methyl, *n*-butyl, phenyl, keto) at one of several locations along 18-carbon fatty acyl chains.



Either both octadecanoyl chains of the phospholipid or only one of them was so substituted. In order to specify a compound in as simple a manner as possible, we adopted a shorthand notation. Thus, the phosphatidylcholine entity, designated PC, is followed by the position of the substituent and its identity (M, methyl; B, *n*-butyl; P, phenyl; K, keto). The numbers preceding PC (1,2 or 2) indicate whether both chains or only the second chain bear a substituent. Two examples illustrate the notation. A complete



listing of the phospholipids (most of them new compounds) is given in Table I. DSPC in Table I and elsewhere refers to the parent lipid 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine.

Experimental Section

Phospholipids. All compounds used in this study, synthesized exactly as described by Menger et al.,[45] were purified by repeated passage over a silica gel (Mallinckrodt) column with a chloroform–methanol gradient in the manner described previously.[45] After each passage, the purity was determined by using thin-layer chromatographic plates (silica gel on polyester backing with 254-nm flurorescent indicator, Aldrich Chem. Co.), which were eluted with chloroform/methanol/water (65:25:4 by volume) and developed with iodine and subsequently stained with Dra-

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gendorff's reagent[46] (Sigma Chemical Co.). Generally, three passes were necessary before the TLC plates were free of noticeable impurities. In some cases, when extraneous peaks were present in the DSC thermogram despite exhaustive chromatography, additional purification was performed. The ^1H and ^{13}C NMR spectra and FAB mass spectra were consistent with the structures shown in Table I.

Sample Preparation. Methanol, used in sample preparation, was 99.9% ACS certified grade, and the chloroform used was HPLC grade which had been filtered through a 0.5-μm filter. Multilamellar suspensions of each lipid were prepared as followed. (1) Lipid (2–4 mg) was dissolved in methanol–chloroform (50/50 by volume) placed in a 25- or 50-mL round-bottom flask. (2) The solvent was then evaporated with a stream of nitrogen, leaving the lipid as a thin film on the walls. (3) Remaining traces of solvent were removed by placing the samples on a vacuum line at 10^{-3} mmHg for 12 h. If this step was deleted, multiple and/or broad peaks were observed, presumably because solvent molecules were not completely removed from the lipids. (4) HEPES buffer (2.0 mL, 10mM, pH 7.5, 160 mM KCl, and 0.1 mM EDTA) was added to the flask containing the film, and the mixture was vigorously vortexed for 5 min with a Vortex Jr. Mixer (Scientific Industries, Inc.). Initially, DSC measurements were performed immediately on suspensions prepared this way to determine the approximate position of any thermal transitions. In the final DSC measurements, all samples were, in addition, stirred at 10 °C above the main phase transition for 30 min to ensure bilayer hydration. When the suspensions had thermal transitions near 0 °C, the suspensions were (in addition to protocol described above) prepared in a 50% aqueous ethylene glycol solution (w/w). This permitted a longer base line prior to the transition. The scans in both systems, aqueous buffer and aqueous ethylene glycol, are given in Figure 1. Lipid concentrations in samples taken from the calorimeter were determined by a phosphate analysis described below.

Phosphate Analysis.[47,48] Magnesium nitrate hexahydrate and ammonium molybdate(VI) tetrahydrate were purchased from Aldrich Chemical Co. Ascorbic acid was obtained from J. T. Baker Co. A Bausch & Lomb Spectronic 2000, which was calibrated with a holmium oxide glass filter, was used to read all absorbances. Standard curves were made from solutions with known concentrations of KH_2PO_4; absorbances were determined relative to a blank composed of all components except a source of phosphate. As soon as a DSC run was completed, the suspension was agitated to ensure uniformity by drawing it in and out of a syringe several times. An aliquot (100 μL) was then transferred immediately to each of eight screw cap test tubes to which was added 0.3 mL of 5% magnesium nitrate in 95% ethanol solution (prepared fresh daily). After evaporation to dryness, the tubes were heated over a Fischer burner until the emission of brown fumes had subsided. Each tube, after receiving 0.6 mL of 0.5 N HCl, was capped and heated in a boiling water bath for 30 min. Next, the tubes received 1.4 mL of a solution containing six parts of 0.42% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 1 N H_2SO_4 and one part of 10% aqueous ascorbic acid. This was followed by capping, vortexing for 15 s, and incubating at 45 °C for 30 min. A 1.0-mL aliquot of this solution was diluted to 5.0 mL with deionized water and vortexed for 30 s and the absorbance read at 820 nm. Phosphate concentrations were found to be in the 0.9–3.2 ± 0.06 mM range. No difference was observed when phosphate analyses were carried out before and after the DSC runs.

Differential Scanning Calorimetry. All calorimetric measurements were performed with a MC-2 differential scanning calorimeter purchased from Microcal Inc. and interfaced to an IBM personal computer. The calorimeter consisted of two cells, one for the sample and one for the buffer or solvent (reference). The cells were heated at a constant rate and in such a way that zero temperature difference was maintained between the two. When a phase change occurred, more heat was added to the sample cell in order to keep the temperature constant in both cells. This excess power applied to the sample cell, recorded digitally at uniformly spaced temperature intervals, was proportional to the excess heat capacity. A heating rate of ~50 °C/h (and in a few cases 12 °C/h) was used in the experiments, which were carried out from 1 to 80 °C for suspensions prepared in buffer and from −5 to 30 °C for suspensions prepared in 50% ethylene glycol–water. Data points (temperature and power) were recorded every 2 s for the sharper transitions and every 5 s for the broader transitions. After each experiment, the cells were rinsed several times with water and methanol and then dried in vacuo.

Thermograms were initially converted from millicalories/minute to millicalories/degree by dividing by the scan rate at each point. A buffer vs buffer base line, also normalized to millicalories/degree, was subtracted from each thermogram to eliminate variations due to any mismatch between the two cells. Finally, individual data points were divided by the number of millimoles of lipid, determined from the phosphate analysis, thereby converting the data to millicalories/degree·mole. All curves were filtered digitally by the splines method. Thus, normalized excess heat capacity curves for transitions were established by connecting the base line before and after the transition by the method of splines and by performing a point by point subtraction. The calorimetric enthalpy change, ΔH_{cal}, was determined via a numerical integration, while the van't Hoff enthalpy was calculated from eq 1 (assuming a two-state

$$\Delta H_{vH} = [(\langle \Delta C_p \rangle)_{max} 4RT_c^2]^{0.5} \quad (1)$$

model).[49,50] ΔC_p is the excess heat capacity and T_c is the temperature at the maximum in the excess heat capacity profile. The cooperativity unit (CU) was calculated from eq 2. In those cases where the thermogram showed multiple transitions, the curves were deconvoluted by a curve-fitting program (Microcal, Inc.) based on a nonlinear least-squares minimization.

$$CU = \Delta H_{vH}/\Delta H_{cal} \quad (2)$$

Table II. Thermodynamic Characteristics of Alkyl Branched Diacylphosphatidylcholines[a,b]

	lower transn		main transn			
PC	T_c	ΔH_{cal}	T_c	ΔH_{cal}	CU	ΔS
DSPC	52.3	1.6	54.8	10.6	198 (319)[c]	32
(1,2)-PC-4M			30.1	7.9	160	26
(1,2)-PC-6M			7.2	5.1	137	18
(1,2)-PC-16M	16.2	2.9	26.9	7.5	54	25
(2)-PC-4M			41.5	8.8	175	28
(2)-PC-6M			30.5	6.5	104	21
(2)-PC-8M			18.6	5.1	62	17
(2)-PC-10M			6.1 (5.6)[d]	3.5 (3.7)[d]	28	13
(2)-PC-12M			8.0 (8.7)[d]	5.7 (5.7)[d]	20	20
(2)-PC-16M			38.5	9.8	76	31
(1,2)-PC-4B			19.8	9.5	81	32

[a] Units: T_c, °C; ΔH_{cal}, kcal/mol; ΔS, cal/mol·deg K. [b] Phospholipids were prepared from racemic fatty acids. [c] The 198 value was obtained from commercial DSPC (Avanti) used as received. The 319 value was obtained from commercial DSPC purified by column chromatography (see Experimental Section). Purification had no effect on T_c and ΔH_{cal}. Synthetic branched phospholipids in the table were all purified by chromatography (always twice and usually three times). [d] Values in parentheses were obtained from runs carried out in 50% aqueous ethylene glycol (w/w).

Results

It should be stated at the outset that our work focused primarily on the $P_{\beta'} \rightarrow L_\alpha$ process (the so-called "main" transition known to represent "melting" or disordering of the hydrocarbon tails). Our objective was to relate structural perturbations of the chains to the properties of the main transition which is, by far, the most widely studied thermodynamic event in bilayer chemistry. "Pretransitions", although very interesting,[35] have been recorded here only for the sake of thoroughness, and they are not interpreted on a molecular level. Conceivably, minor transitions not detected at our 50 °C/h scan rate might have been detected at slower rates. On the other hand, no observable dependence of the main transition on scan rate was observed. Thus, the transition temperature T_c for DPSC changed an insignificant 0.05 °C when the scan rate was diminished from 50 °C/h to 12 °C/h, and ΔH_{cal} did not change at all. The reason for the relatively fast scan rate, apart from convenience with our multitude of compounds, was that it gave an improved signal-to-noise ratio, an important item when only small amounts of synthetic lipid (prepared by multistep sequences) are available.

High-sensitivity DSC heating thermograms of DSPC show a sharp peak at T_c = 54.8 °C with ΔH_{cal} = 10.6 kcal/mol and CU = 319 (Table II) in addition to a pretransition. These values agree well with those of Mabrey and Sturtevant[17] and Lewis et al.[18] especially in view of the notorious sensitivity of the parameters to impurities.[51] As mentioned in the Experimental Section,

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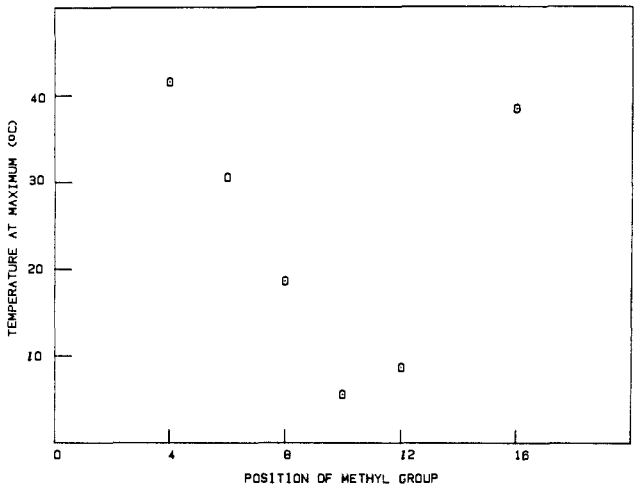

Figure 2. T_c vs position of the methyl group for the six lipids in Figure 1.

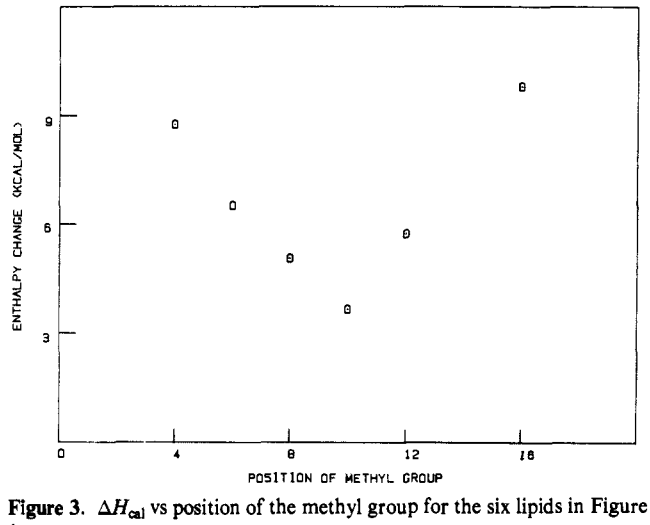

Figure 3. ΔH_{cal} vs position of the methyl group for the six lipids in Figure 1.

extreme care was taken to secure phospholipids of high purity.

A single methyl substituent at any of six positions on the second chain broadens the main gel-to-liquid crystal transition and shifts it to lower temperatures (Figure 1 and Table II). The effect of a methyl branch can be considerable. Thus, the 10-methyl group in 2-PC-10M lowers T_c by 49.2 °C and ΔH_{cal} by 6.9 kcal/mol relative to those of the parent, DSPC. Indeed, one can barely detect a transition for 2-PC-10M. On the other hand, 2-PC-4M retains a sharp peak found with DSPC. When T_c and ΔH_{cal} are plotted vs the substitution site of the methyl group (Figures 2 and 3), a sharp minimum is seen near position 10. The dramatic difference between center and termini is reminiscent of the behavior observed with positional isomers of unsaturated lecithins.[32]

Transition cooperativity, CU, is likewise reduced by a methyl group near the center relative to the two ends of the acyl chain (Table II). CU values can be viewed as the average number of molecules that melt cooperatively in a two-state transition. Our measurements show the size of the cooperative unit decreases from 319 to 76 to 28 as the lipid is changed from no methyl (DSPC) to a 16-methyl to a 10-methyl on the second chain, respectively. Note that CU values are based on a two-state model (see eq 1 and 2) that requires symmetric peaks. Since many of the lipid peaks are not symmetric, the resulting CU values are approximate and useful only to indicate qualitative trends among the lipids. No conclusion at the molecular level will be based on absolute CU data.

When both acyl chains possessed methyl branches, transitions could not be observed for (1,2)-PC-8M, (1,2)-PC-10M, and (1,2)-PC-12M even when scans were initiated at −11 °C. In other words, if these compounds have transitions, they must take place

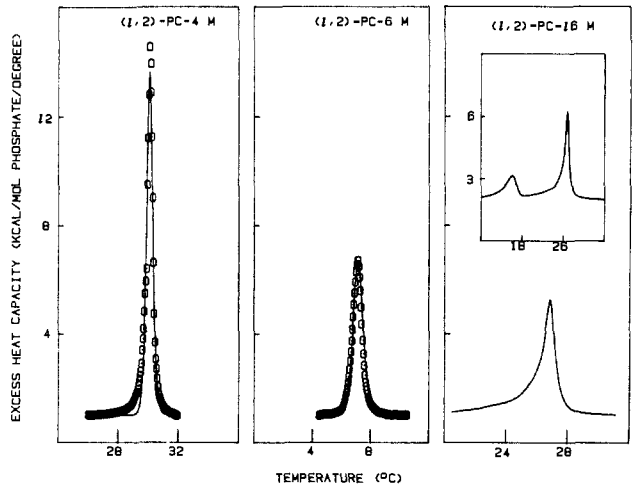

Figure 4. DSC thermograms for three lipids substituted with two methyl groups (one on each 18-carbon chain) at the 4, 6, and 16-positions. The solid lines indicate the experimentally determined data and the circles show curves derived from a computer program based on a two-state model.

below −11 °C. DSC profiles for the other doubly methylated lipids are shown in Figure 4. For (1,2)-PC-4M and (1,2)-PC-6M, excellent fits were obtained with a nonlinear least-squares program based on the analysis of Sturtevant,[52,53] the purity estimated from these analyses is greater than 99.9% for both compounds. For (1,2)-PC-16M, a minor $L_c \rightarrow L_\beta$ transition[35] was evident (see inset in the third panel of Figure 4). The parameters derived from the DSC profiles are listed in Table II. Not surprisingly, at any given position two methyls are more disruptive than a single substituent. For example, (1,2)-PC-6M and (2)-PC-6M have T_c values of 7.2 and 30.5°C, respectively. The T_c = 18.6 °C for (2)-PC-8M can be contrasted with a $T_c < -11$ °C (the minimum temperature in the DSC scans) for (1,2)-PC-8M.

Nuhn et al.[40] found a transition for (1,2)-PC-4M [designated 1,2-di(4C_1-18:0)PC] at T_c = 29.5 °C (ΔH_{cal} = 8.37 kcal/mol. In Table I we report for this compound a T_c = 30.1 °C (ΔH_{cal} = 7.9 kcal/mol), which is in good agreement. McElhaney et al.[35,37] detected for (1,2)-PC-16M (designated 19ai PC) a $L_c \rightarrow L_\beta$ transition (T_c = 15.6 °C and ΔH_{cal} = 2.9 kcal/mol) and a main gel-to-liquid crystal transition (T_c = 29.5 °C and ΔH_{cal} = 7.9 kcal/mol). Our data on (1,2)-PC-16M in Table II are similar except for the main transition T_c, which is 2.6 °C lower than theirs. Repeated chromatography did not rectify the small discrepancy which could be due to an impurity in one of the samples or to a difference in sample preparation.

A trend for T_c of doubly methylated lipids exists similar to that observed in Figure 2, i.e., T_c is systematically lowered as the methyl groups are moved toward the center of the chain. By extrapolation one can estimate roughly a T_c = −16 °C for (1,2)-PC-8M and a T_c = −39 °C for (1,2)-PC-12M. If (1,2)-PC-10M also follows the trend, its ΔH_{cal} would be close to zero, and the transition would be indiscernible.

For the butylated and phenylated lipids, a transition was found only for (1,2)-PC-4B (reinforcing the notion, apparent in Figure 1, that groups near the head-group are the least disruptive). The DSC profile for (1,2)-PC-4B in Figure 5 has a shape similar to that observed for (1,2)-PC-4M, but the T_c is almost 20 °C lower. This difference is largely entropic in origin (Table II).

Remarkable behavior was observed for all five diketo-substituted DSPC derivatives listed in Table I. Each of them undergoes multiple transitions in the 50–60 °C region, and at least one transition takes place in the 35–45 °C interval, thus indicating the presence of a complex set of thermal events. Since some of the transitions overlap, the digital data were subjected to a non-

(52) Sturtevant, J. M.; Ho, C.; Reimann, A. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2239.
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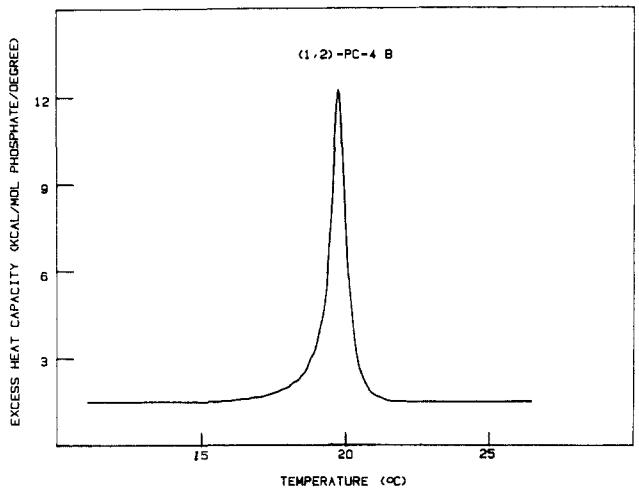

Figure 5. DSC thermograms for a phosphatidylcholine substituted with two butyl groups (one on each 18-carbon chain) at the 4-position.

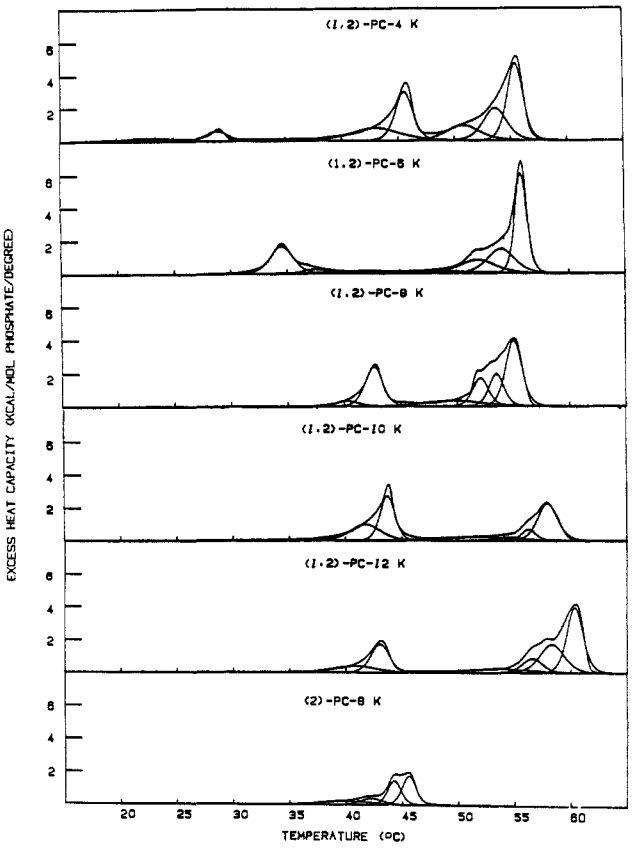

Figure 6. DSC thermograms of various keto-substituted distearoylphosphatidylcholines showing results of deconvolution.

linear least-squares curve-fitting program based on an independent, two-state model. The parameters found for the final fits are listed in Table III, and the curves for the individual transitions are plotted in Figure 6. At least six separate transitions were required to complete the deconvolutions. A complex DSC profile was also generated by the only monoketo lipid investigated, (2)-PC-8K. Again, we were able to fit the digital data to overlapping, two-state transitions, the results of which are given in Table III. Many of the keto lipid transitions are, probably, solid-state changes where equilibrium conditions throughout the DSC experiment have not been achieved. No attempt was made to identify the extraordinary array of transitions imparted by the seemingly innocuous keto functionality to the lipids. Nonetheless, their presence is worth noting because one would thereby predict, for example, that a keto group should substantially affect other membrane properties (i.e., permeability), a prediction we are in the progress of testing.

Table III. Thermodynamic Data for Keto Diacylphosphatidylcholines[a]

compd	transn no.	T_c	ΔH_{cal}	ΔH_{total}
(1,2)-PC-4K	1	22.6	1.1	30.8
	2	28.9	1.6	
	3	43.0	4.5	
	4	45.6	6.0	
	5	50.8	3.6	
	6	53.6	5.4	
	7	55.4	8.6	
(1,2)-PC-6K	1	34.6	4.5	25.2
	2	37.5	0.8	
	3	45.2	3.0	
	4	51.9	3.5	
	5	54.0	4.9	
	6	55.8	8.5	
(1,2)-PC-8K	1	40.3	0.9	22.3
	2	42.6	4.9	
	3	49.6	2.5	
	4	52.1	3.2	
	5	53.5	3.4	
	6	55.0	7.4	
(1,2)-PC-10K	1	41.8	3.6	17.6
	2	43.8	4.7	
	3	51.2	2.2	
	4	54.5	0.7	
	5	56.3	1.2	
	6	58.0	5.2	
(1,2)-PC-12K	1	40.7	2.1	22.7
	2	43.1	3.7	
	3	53.2	2.1	
	4	56.6	2.2	
	5	58.3	5.1	
	6	60.4	7.5	
(2)-PC-8K	1	39.7	1.6	8.0
	2	42.3	1.1	
	3	44.3	2.4	
	4	45.6	2.9	

[a] The peaks in this table were obtained from the deconvolution of the corresponding calorimetric profiles.

Discussion

The gel phase of aqueous bilayer suspensions (below T_c) is characterized by stiff, extended, and parallel chains in a "crystalline" array where rotational disorder about the carbon–carbon bonds is limited. On the other hand, the liquid–crystal phase (above T_c) consists of bilayers with a nonlinear "fluidity gradient".[54-57] Thus, from the ester carbonyl carbon to about C_{10}, the chains are relatively confined; from C_{10} to C_{18} the molecular motion increases rapidly and uniformly. In contrast, we find that an alkyl substituent is far more disruptive at the center than at either terminus. The problem is, of course, to reconcile these sets of observations. In order to do so, we must briefly review relevant information on membrane structure.[58]

Two major energy components are responsible for energy absorption at the main phase transition: a reduction in van der Waals interactions among the chains and an increase in the number of gauche C–C linkages.[59] Other terms have been identified, but these are either insignificant or else most likely will cancel when comparing isomeric derivatives of DSPC. As T_c is exceeded, the average number of kinks per molecule[54,60] increases from 0 to 2–6 (Raman data ostensibly point to even larger number of gauche conformations[61]). According to spin label data,[57] the probability

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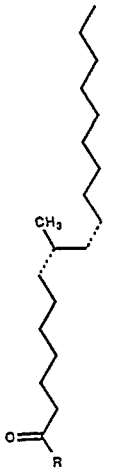
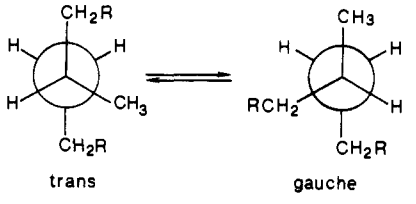


Figure 7. 8-Methylstearoyl chain showing a 2g1 kink induced by a methyl substituent.

of a nontrans conformation markedly increases beyond the midpoint of the chain. During the order-to-disorder transition, a bilayer increases its molecular area[62] by 6 Å^2 while concurrently decreasing its thickness[63] by 20% and increasing its head-group hydration by several water molecules per lipid.[62]

Nagle[63] has shown that the destruction of attractive van der Waals interactions upon bilayer melting contributes ~5.5 kcal/mol to the transition enthalpy, a significant fraction. Hence, a methyl substituent could conceivably lower T_c and ΔH_{cal} by "spreading" the chains in the matrix and reducing the total number of van der Waals contacts. Additionally, a methyl substituent can induce a trans-to-gauche transformation shown in eq 3 and



(3)

Figure 7. Force field (MM2) calculations revealed that an isolated chain would exist as 51% trans and 49% gauche. Of course, this trans/gauche ratio (reflecting only intramolecular forces acting upon the potential function) need not be retained in a membrane where neighboring molecules impose additional constraints. Nonetheless, to the extent that gauche conformations contribute, a methyl group will disrupt membrane structure. The disruption can be less than that implied in eq 3 because a second gauche rotation, to give a so-called 2g1 kink,[60] partially reestablishes linearity. Such a 2g1 kink, depicted schematically in Figure 7, is formed from an all-trans chain upon rotating one C–C bond by +120° and either of the two next nearest C–C bonds by −120°. As a consequence, the overall chain length decreases 1.3 Å and the molecular volume increases 25–50 Å^3. In summary, the methyl group can be viewed as a "kink-inducing unit" that, as with the cis double bond, provides an inherent bend.[32] In contrast to the rigid double bond, however, the methyl group permits a degree of conformational flexibility at the site of disruption.

A final point will be made by way of introduction. Clearly, one must assess the effect of a chain substituent on *both* sides of the gel-to-liquid crystal equilibrium. The importance of this obvious but seldom-mentioned notion can be appreciated by considering, for example, an alkyl substituent that spreads the chains apart at a particular locus in the low-temperature phase. If, however, the perturbation persists in the high-temperature phase, then the effect on observed thermotropic properties will be minimal. In other words, large decreases in T_c will occur only

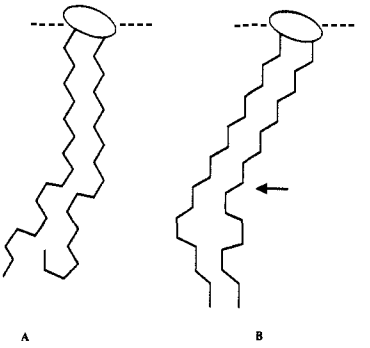


Figure 8. Two schematic representations of the liquid crystalline phase of DSPC. In model A, the first halves of the chains are linear and perpendicular to the bilayer surface (dotted line); rotameric disorder occurs in the distal halves of the chains. In model B, the first halves of the chains are linear but oblique to the bilayer surface; disorder develops beyond the midpoints of the chains. See text for arguments favoring model B. The arrow indicates the region near the statistical bend where methyl substitution imparts its major effect on the thermotropic parameters.

when a substituent destabilizes the gel phase but not the liquid crystalline phase or, alternatively, stabilizes the liquid crystalline phase but not the gel phase.

With this background, it is now possible to address the central question of why a methyl group near the center induces the maximum perturbation of T_c and ΔH_{cal} (Figures 2 and 3). In all likelihood, the close-packed matrix within the gel enforces a rather normal all-trans conformation upon the chains despite methyl substituents. Although a methyl group undoubtedly expands the molecular cross section (by as much as 50% according to our MACROMODEL calculations on 8-methylstearic acid[45]), it is difficult to conceive of a highly ordered gel phase accommodating a large static population of gauche conformations, or even 2g1 kinks, all focused at one particular site. Only near the free-methyl termini is rotameric disorder possible in the gel state.[63] If indeed the chains within the gel remain primarily linear, parallel, and tightly packed, then there is no obvious explanation for why a methyl substituent at the chain center should have a much more dramatic effect than one at either end. Thus, we must search for an explanation at the "right-hand" side of the transition equilibrium, namely, the liquid crystalline state.

As already mentioned, above T_c the carbons in the first half of the bilayer chains (i.e., those proximal to the head-group) are immobilized relative to the second half. This crucial observation, which must be reconciled with our data, cannot be explained by a model in which the area-per-molecule expands above T_c, thereby allowing linear chains (roughly perpendicular to the bilayer surface) to "curl" along the second half (Figure 8a). The problem here lies in the fact that such a process would require a higher chain density at the free-methyl end than at the head-group, a conclusion that few people are ready to accept.[63] Moreover, one would be forced to fill the empty spaces surrounding the first half of the chains with water (leaving the second half free to contract). But recent NMR data of ours[64] showed quite clearly that static water is absent, or nearly so, along the entire chain.

A more plausible (but unproven) explanation for the "fluidity gradient" was published by McFarland and McConnell.[65] According to their hypothesis, lipid chains are endowed with a "statistical bend" near their center, thereby dividing the chains into two segments of roughly equal length (Figure 8b). The segment nearer to the head-group lies at a 30° angle to the membrane surface; the more distal segment lies perpendicular to the surface. Although not intuitively obvious, a central bend provides the second half of the chain ~12% more space within which to maneuver.[65] This may appear to be a small variation,

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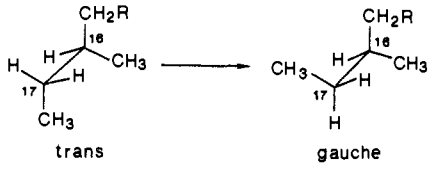
but it must be remembered that melting hydrocarbons increase their density by only 10–15%, so that a 12% differential is significant.

The role of the methyl substituent can be qualitatively understood in terms of the statistical bend hypothesis. A methyl near the center of the chain, as in (2)-PC-10M, stabilizes the critical bend that, ostensibly, develops when a gel reorganizes into a liquid crystalline phase. Hence, a centrally located methyl reduces appreciably T_c and ΔH_{cal} relative to DSPC and to DSPC substituted at other loci (Figures 2 and 3). Perturbations of a methyl near the chain terminus, as in (2)-PC-16M, are relatively modest. Since the chain segment between the crucial central bend and the terminal methyl permits the segment to rotate along the locus of a cone (with the kink at the apex), interchain space is, of all substitution sites, most available near the terminal methyl to accommodate branching and kinking. The behavior of (2)-PC-16M is thus rationalized. Note also that a methyl-induced kink near the terminus has a small "leverage" effect in that only a short segment is skewed out of position by a torsional displacement (eq 4). A 16-methyl group therefore creates far less packing distortion than a 10-methyl.



(4)

As seen in Figure 1 and Table III, methyl substitution at carbon 4 has the sharpest signal of all DSC scans as well as T_c and ΔH_{cal} values nearest to those of the parent DSPC. In addition, only (1,2)-PC-4B, of all the butylated lipids listed in Table I, has an observable T_c (the others being too low to measure). Yet the C-4 is known to be more ordered and immobilized than carbons beyond the chain center.[54-57] One might have predicted (for the same reasons advanced to explain why a 16-methyl is relatively innocuous) that a 4-methyl should be the *most* disruptive, whereas the opposite was found experimentally. Apparently, the 4-methyl in eq 3 remains gauche to the chains in order that the chain components retain their trans relationship. To do otherwise would impose a serious perturbation in a region where the chains are anchored by the head-group and motionally restricted in an environment equivalent to a highly viscous liquid.[59] Since, therefore, conformational and motional properties at C-4 for the liquid crystalline phase are similar to those for the gel phase, methyl substitution at C-4 has a relatively minor effect on the thermotropic behavior. As stated above, a substituent will create thermotropic perturbations only when it has a disparate effect on the two phases.

According to the above model, a methyl branch can be regarded as a kink-inducing entity, both in the gel and liquid crystal phases. Whether or not the methyl group so functions depends upon location. Near the head-group, the chains remain linear despite the methyl. Near the center, a methyl induces a kink essential to the melting of the chains above T_c. At the termini, kinking can readily occur, but the impact on membrane behavior is modest. It is in this manner that the "V-shaped" plots in Figures 2 and 3 can be interpreted.

Thermodynamic parameters in Table II are consistent with the model. The main phase transition of DSPC is associated with ΔH_{cal} = 10.6 kcal/mol and ΔS = 32 eu. A 4-methyl reduces these values to ΔH_{cal} = 8.8 kcal/mol and ΔS = 28 eu. These small changes are indicative of relatively minor relocations in the first half of the chain during the melting process; a 4-methyl group, consequently, has little impact upon the thermodynamic parameters. In contrast, dramatic reductions were noted with a 10-methyl (ΔH_{cal} = 3.7 kcal/mol and ΔS = 13 eu). Thermodynamic parameters for (2)-PC-16M are more normal because, as mentioned, substitution near the terminal methyl interferes little with the conformational changes focused near the center of the chains. Cooperativity units, CU, in Table II (equated roughly with the number of molecules that melt in unison) follow the same trend as do the thermodynamic parameters.

In a sense, we have used the methyl group as a probe. By varying the probe position, and by assuming that the probe should exert its greatest effect at the site of cooperative bending, we are able to locate this site. The evidence points strongly to the midchain region.

Not surprisingly, branching at both chains causes more disorder in the bilayer than a single substitution (e.g., T_c = 7.2 and 30.5 °C for (1,2)-PC-6M and (2)-PC-6M, respectively). No transitions were observed above −11 °C for any butylated or phenylated lipids in Table I except with (1,2)-PC-4B. Butyl and phenyl groups should drive the trans/gauche equilibrium in eq 3 to the right more effectively than a methyl group. In addition, the chains, forced apart by the bulky substituents, should suffer reduced van der Waals contacts. Repulsion among neighboring substituents can, of course, be mitigated by interdigitation (requiring only slight irregularities in the smoothness of the membrane surface). Despite this, ΔH_{cal} for (1,2)-PC-4B is unexpectedly high (only 0.9 kcal/mol less than that of DSPC). Whatever the disruptions generated by the 4-butyl, they are not effectively transmitted "further down the line" during the melting process, once again affirming the apparent rigidity of the initial chain segments.

Our results for the keto lipids (Table III) are complicated. The position of the keto groups is not as critical as it is with the methyls. Converting a methylene to a carbonyl actually enhances the enthalpic requirements for melting (e.g., ΔH_{total} = 22.3 kcal/mol for (1,2)-PC-8K compared to ΔH_{total} = 12.2 kcal/mol for DSPC). No doubt intramolecular and intermolecular dipole–dipole interactions contribute to the transition enthalpy by helping stabilize the bilayer matrix. One is reminded here of the "hydrogen belt" region of Murakami et al.[66] who inserted amide groups within the chains to promote bilayer formation. When there exists a single carbonyl per lipid, carbonyl–carbonyl attraction is reduced by dilution [ΔH_{cal} is only 8.0 kcal/mol for (2)-PC-8K]. At present, the multitude of transitions for each lipid in Table III is not understood on a molecular level. Additional studies at lower scan rates seem warranted.

Systematic structure–behavior studies, such as presented here, will be important in developing and testing membrane models. The thermotropic properties elaborated above are also a prerequisite for analyzing transport rates which we are currently relating to lipid structure. In 1884 it was written about membranes, "Their varied functions are the result of the collusion of radicles of strongly contrasting properties".[1] Perhaps, through the use of synthetic organic chemistry, the "radicles" can be varied and the collusion exposed so that the functions become controllable for specific purposes.

Acknowledgment. This work was supported by NIH Grant No. 5-21423 (F.M.M.) and NSF equipment Grant No. DMB-8500319 (H.P.H.).

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