Biochimica et Biophysica Acta 863 (1986) 33–44
Elsevier

BBA 73165

Dilatometric studies of isobranched phosphatidylcholines

C.P. Yang [a], M.C. Wiener [a], R.N.A.H. Lewis [b], R.N. McElhaney [b] and J.F. Nagle [a]

[a] *Departments of Physics and Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213 (U.S.A.) and* [b] *Department of Biochemistry, The University of Alberta, Edmonton, Alberta T6G 2H7 (Canada)*

(Received 5 June 1986)

Key words: Lipid; Branched lipid; Dilatometry; Odd/even effect; Phase transition; Bilayer structure

Absolute apparent specific volumes have been obtained for phosphatidylcholine lipids with saturated, isobranched hydrocarbon chains with *n*i = 15 to 20 carbons, with an emphasis upon phase transition behavior, both equilibrium and kinetic. The temperature of the chain-melting transition extrapolates with increasing chain length to the melting temperature of polyethylene with a small odd/even alternation. There are also odd/even alternations in the volume of transition and in the hysteresis of the chain-melting transition, but with the odd and even reversed when compared with the larger odd/even alternation in the lower solid-solid transition that occurs in the longer chain *n*i lipids. A phenomenological picture is given for the coalescence of the two transitions for shorter *n*i lipids and this picture is used to sharpen the discussion of the kinetic mechanism of melting. A temperature-reversal experiment shows that the melting from the lowest temperature crystal or C phase to the fluid F phase does not proceed via the metastable gel G phase for 16i. The dilatometric results are combined with recent X-ray structural results for the C and G phases of 17i and 20i to deduce various structural information, including the hydration numbers and the volume of the headgroup, $V_H = 341$ Å^3, which agrees very well with V_H for straight-chain phosphatidylcholines. For the chain-melted F phase the assumption that the methylene volumes of the different *n*i lipids should be the same at the same temperature is used to obtain the volumes of the methylene and the methyl groups.

Introduction

The thermotropic phase behavior of phosphatidylcholine (PC) model membrane systems is of considerable interest from both the physical and biological perspectives. Most of the available data on PC model membranes have been obtained for saturated *n*-acyl-PCs which have been characterized by a variety of physical techniques [1]. In recent years there has been growing interest in PC model membrane systems containing iso- and anteiso-branched fatty acyl groups, mainly because these fatty acyl chains are abundant and widespread constituents of membrane lipids of bacterial microorganisms [2]. Of these, the most thoroughly studied to date are the 1,2-di-isoacyl-PCs, which, when compared to the straight chain acyl chains, involve a substitution of a methyl group for one of the hydrogens on the penultimate carbon on each acyl chain. These lipids have been characterized by differential thermal analysis [3],

Abbreviations: PC, any lipid with *sn*-glycero-3-phosphocholine headgroup; *n*i, PC lipids with 1,2-diisoacyl hydrocarbon chains, where *n* denotes the number of carbon atoms in each isoacyl chain; lipid phases: C is crystal, G is gel and F is fluid, i.e. liquid-crystalline; DSC, differential scanning calorimetry.

Correspondence: Dr. J.F. Nagle, Departments of Physics and Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213, U.S.A.

DSC [2], Fourier transform infrared spectroscopy and ^{31}P-magnetic resonance [4], X-ray diffraction [5] and by monolayer film balance techniques [6,7]. Such studies showed that the thermotropic phase behaviour of the isoacyl-PCs consists of two main events. One involves a solid-solid type of transition from a 'crystal' C phase to a gel G phase, and the other is the conventional chain-melting transition from the gel to the fluid liquid-crystalline F phase. When compared with the saturated *n*-acyl-PCs, these compounds exhibit lower chain-melting phase transition temperatures and a greater tendency to form C phases. In addition, the X-ray diffraction studies indicate that there are structural differences between the C phase of the odd-numbered and the even-numbered isoacyl-PCs and that the acyl chains of the even-numbered membranes are strongly tilted to the bilayer normal. These structural differences probably account for the odd/even alternation in the properties of the C phase of the isoacyl PCs that were observed in Refs. 2 and 4. (For a general discussion of the odd/even effect for alkanes, see Ref. 8.)

The DSC studies showed that there was considerable hysteresis in the transition from the C phase to the G phase, especially in the case of the odd-numbered compounds. It was also apparent that the phase behaviour of the shorter-chain compounds, for which the two transitions are not demonstrably separable in temperature, was different from that of their longer-chain counterparts, for which two distinct transitions are calorimetrically observable in both the heating and cooling modes [4]. These differences raised some interesting mechanistic questions which could not be easily addressed by the physical techniques used. Dilatometry is a non-invasive observation of the temperature-dependent, specific volume changes and has been very useful in the characterization of the thermotropic phase behaviour of lipid bilayer systems [9,10]. In addition dilatometry can be performed with extremely slow heating and cooling rates and this has been exploited in the characterization of metastable behaviour and some processes which are prone to hysteresis [11,12]. A dilatometric characterization of the thermotropic phase behaviour of the isoacylphosphatidylcholines is presented here. The absolute apparent specific volumes that are reported here were also used to advantage in extracting more information from the X-ray structural data reported by Church et al. [5].

Materials and Methods

The synthesis of 1,2-di-isoacylphosphatidylcholines is described in Ref. 4. The dilatometer has been described in Ref. 13, and it is capable of scanning from 0 to 10 K per hour in both heating and cooling modes as well as operating isothermally. Each lipid sample was thoroughly dried in a vacuum oven (55 °C), weighed, and then dispersed in water by stirring or gently vortexing at temperatures 10 to 20 K higher than the main chain-melting transition temperature. After the formation of an apparently homogeneous dispersion, the sample was brought through the main transition temperature at least four times by cycling between an ice water bath and a hot water bath. The lipid concentrations ranged from 1.5 mg/ml to 5 mg/ml in the dilatometric studies of these lipids. The samples were constantly stirred in the dilatometer.

During the course of the experiments, the GF transition half-width was monitored. Excessive broadening of the half-width indicates sample contamination and/or degradation. For all the reported results the half-widths increased by less than 20% when compared to the fresh sample. In addition, within experimental error, transition temperatures and volume changes showed no systematic variation in successive scans. These observations indicate that no substantial degradation occurred in the samples over the time-course of the experiment.

To investigate the kinetic mechanism of some of the transitions, we developed a procedure used with the dilatometer that we shall call temperature reversal. In temperature-reversal experiments, the temperature of the sample was first increased in a heating scan. Part way through a phase transition the temperature increase was stopped and the temperature was then decreased in successive near-equilibrium steps. A control experiment was performed on samples at temperatures at which no transition occurs; this showed that the apparent volume changes due to instrumental

artifacts introduced by this reversal process are negligible compared to the volume changes observed when a transition is present.

Absolute apparent specific volumes were determined at certain convenient temperatures, such as 4°C, 37°C and room temperature by a buoyancy technique [9]. Lipid was dispersed in several $H_2O/^2H_2O$ mixtures, then centrifuged. Upper and lower bounds on the specific volume were determined by observing the highest concentration of ^2H_2O at which the lipid sank and the lowest concentration of ^2H_2O at which the lipid floated, respectively. Errors were typically ± 0.002 ml/g.

Differential scanning calorimetry was carried out on a Microcal MC-1 calorimeter. All heating scans were at rates of 10–12 K/h, with sample concentrations of 4–6 mg/ml.

Results

The absolute apparent specific volumes for the isobranched phosphatidylcholines *n*i, where *n* ranges from 15 to 20 in these dilatometric studies, are shown in Fig. 1. In agreement with earlier studies [2], upon heating there are two distinct transitions in 17i, 19i and 20i. The phase at lowest temperatures is called the C phase; it undergoes a transition at T_{CG} to a gel phase called the G phase, which in turn undergoes a transition at a higher temperature, T_{GF}, to a fluid, chain-melted phase called F. Upon cooling there is a small amount of hysteresis in the T_{GF} transition and much more in the T_{CG} transition, as indicated in Fig. 1. For 16i we did not observe two distinct transitions but from analysis of the total enthalpy of transition [2], from infrared studies [4], and from our measurements of the volume of transition, it is clear that both transitions occur concomitantly, so the transition, which has considerable hysteresis, is between F and C. Previous studies [2] found a similar concomitant transition for 15i and 18i as for 16i. Our present dilatometric studies corroborate that 18i has only one transition going from C to F in heating scans but sometimes 18i has two transitions in slow cooling scans, as shown in Fig. 1. For 15i, we detected only the G to F transition in the dilatometer. Table I lists the salient thermodynamic properties for 15i through 21i.

The equilibrium transition temperatures T_{GF} were obtained as shown in Fig. 2 where the ap-

TABLE I

THERMODYNAMIC PROPERTIES OF ISOBRANCHED PCs

The coefficients of volume expansion α (K^{-1}) are multiplied by 10^4 with errors of about 40 percent. Temperatures are in °C. $\Delta T_{1/2}$ are dilatometric halfwidths [9]. Volume changes are in 10^{-2} ml/g with errors of $3 \cdot 10^{-3}$ ml/g for the GF transitions and about $6 \cdot 10^{-3}$ ml/g for the CG transitions. Transition enthalpies are in kcal/mol.

PC	15i	16i	17i	18i	19i	20i	21i
α_F	12	10				10	
T_{GF}	5.9	18.9	26.7	36.6	43.1	50.4	55.9 [c]
$\Delta T_{1/2}$	0.4	0.1	0.6	0.2	0.5	0.3	0.4 [c]
ΔV_{GF}	3.0	3.6	4.4	4.5	5.1	5.2	
ΔH_{GF}	3 [d]	14.2 [b]	7.1	10 [d]	9.7	11.5	14.8
α_G					15	17	16
T_{CG}		22.8 [b]	16.2	35.0 [a]	26.9	42.7	34 [c]
$\Delta T_{1/2}$		0.4 [b]	2.3	1.1	1.4	0.8	
ΔV_{CG}		4.1 [b]	≥ 2.4	4.7	≥ 1.6	4.5	
ΔH_{CG}	3 [d]	[b]	6.6 [e]	8 [d]	7.8	8.5	≥ 6.4
α_c		4		5	7	4	

[a] T_{CG} of 18i is determined from heating scans; see text.
[b] Believed to be a CF transition.
[c] Determined calorimetrically only.
[d] From Ref. 2.
[e] From Ref. 5.

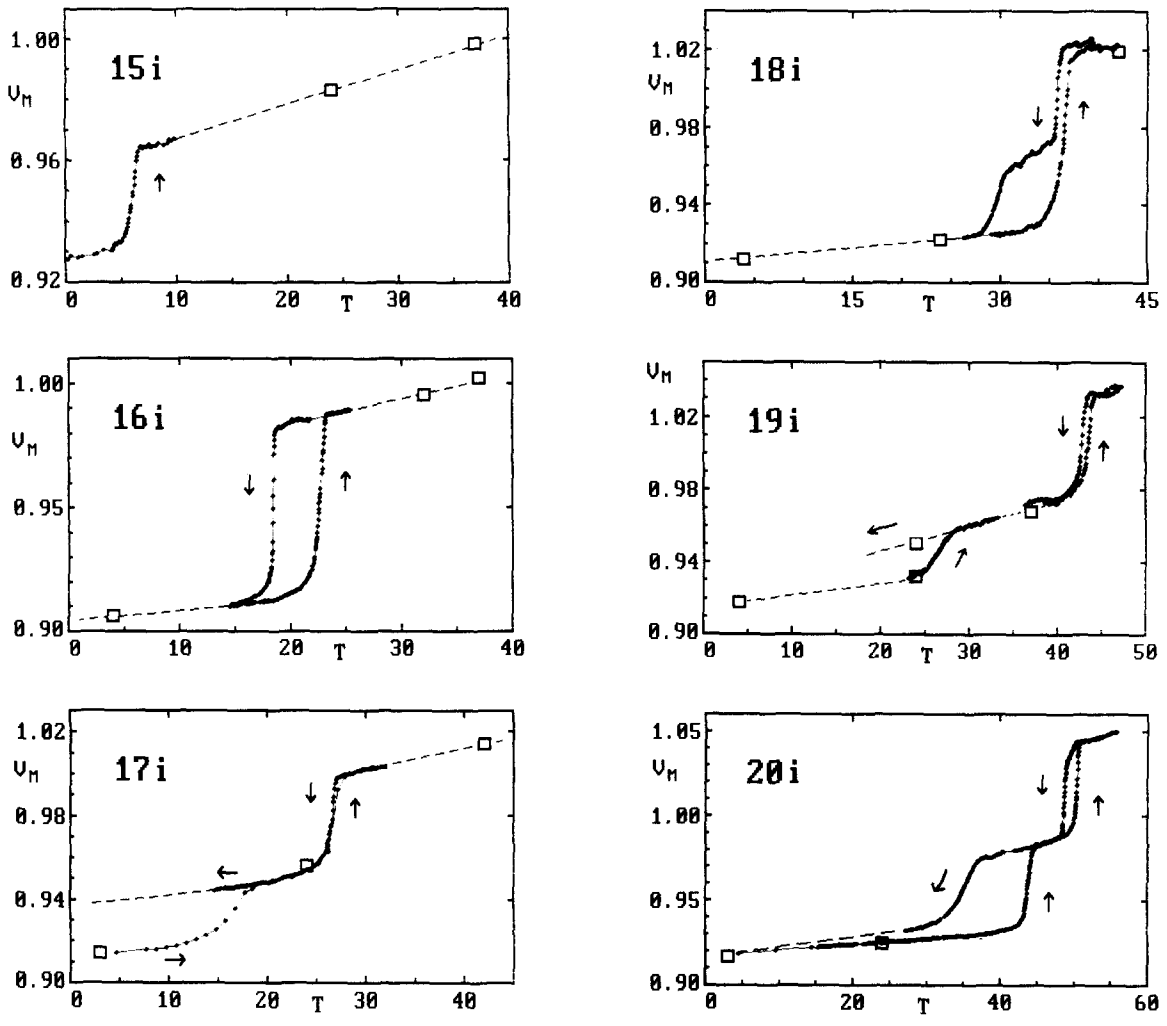

Fig. 1. Absolute apparent specific volumes of isobranched phosphatidylcholines with chain length 15 through 20 as a function of temperature in °C. Open squares are the apparent absolute specific volumes measured by the neutral buoyancy method. The solid dots are the dilatometric scanning data for relative apparent specific volumes changes which have been placed to match up with the absolute specific volume measurements. Dotted lines are the expected extrapolation of the scans. All the scans shown are heating scans unless otherwise indicated by arrows next to the scan. Vertical scales are specific volumes in ml/g of lipid. Scanning rates are typically 1–4 K/h.

parent transition temperature, $T_{GF}(R)$, defined to be the temperature at which half the volume change occurred, is plotted as a function of scanning rate R. The values of $T_{GF}(R)$ in Fig. 2 have already been corrected for the instrumental effect due to the temperature lag of the sample compared to the water bath, in which the temperature is actually measured, so the resulting variations as a function of scanning rate in Fig. 2 reflect the kinetics of the transitions only. For odd values of n, $T_{GF}(R)$ is a nearly linear function of scan rate, so T_{GF} is established straightforwardly. For even values of n there is clearly a residual hysteresis, present even when one extrapolates to zero scanning rate, when heating scans are compared to cooling scans. This residual hysteresis is not the same as the hysteresis indicated in Fig. 1 which is due to nonzero scanning rates. From Fig. 2 (see especially 20i) this residual hysteresis is apparently due to retarded kinetics of formation of G upon

cooling. The values of T_{GF} quoted in Table I are the linearly extrapolated transition temperatures from the positive (heating) scanning rates in Fig. 2.

The values of T_{GF} are plotted in Fig. 3 as a function of $1/(n-\delta)$ where $\delta = 3.7$ was determined by the criterion that the transition temperatures are then best fit by a straight line. The extrapolated temperature intercept obtained for $n = \infty$ is $T_{GF}(\infty) = 149\,^{\circ}C$ which is within error of the melting temperature, 141°C, of long chain polyethylene. In Fig. 4 we plot the differences in T_{GF} from the straight line in Fig. 3. Fig. 4 clearly shows an odd/even effect for T_{GF} with n.

Fig. 5 shows the volume changes ΔV_{GF} at the G to F transition for all the lipids except 16i for which it was not possible to separate ΔV_{GF} from ΔV_{CG}. These volume changes indicate an odd/even effect. A rough extrapolation of the data in Fig. 5 yields a volume change of about 2.1 ml/mole CH_2 for $n = \infty$ in good agreement with the straight-chain PCs [9].

Also shown in Table I are the T_{CG} transition temperatures. These were determined by heating scans with rates ranging from near-equilibrium to 5 K/h. As indicated in Fig. 1 the CG transitions exhibit considerable hysteresis upon cooling. In the case of odd n values long incubation times are required to form the C phase. The incubation time for 17i exceeds 50 h and becomes even slower for 19i and 21i. In this regard there is also a strong

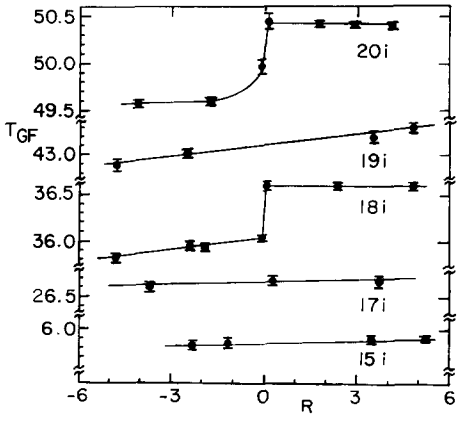

Fig. 2. $T_{GF}(R)$ in °C is the dilatometrically determined midpoint of the GF transition as a function of scanning rates R in K/h. Different lipids are indicated by the numbers below the curve.

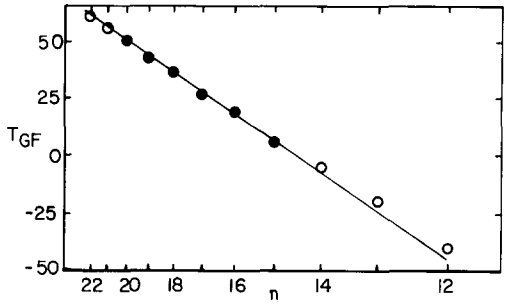

Fig. 3. Transition temperatures T_{GF} in °C as a function of $1/(n-3.7)$. Some values of n are shown. The solid straight line is the function $149.3-1617/(n-3.7)$ which was determined by fitting to the T_{GF} transition temperatures. Solid circles are dilatometric results in this study and open circles are previous calorimetric results [2] for T_{GF}. For the shorter-chain lipids with only one transition upon heating, the values of T_{GF} are the transition temperatures observed upon cooling.

odd/even contrast with the even n lipids requiring much shorter incubation times, being of the order of 5 h for 20i, but with the similarity that the incubation time also appears to decrease with decreasing chain length.

The CG transition in 17i has been subject to considerable scrunity. The transition temperature T_{CG} as determined by heating differential scanning calorimetry has been reported as 15°C for incubation times of the order of a day [2] and this has been confirmed by independent DSC analyses performed in the current study. Upon longer incubation, however, T_{CG} increases to about 19°C, but the kinetics of this change in T_{CG} appear to be very sensitive to the particular sample in ways that

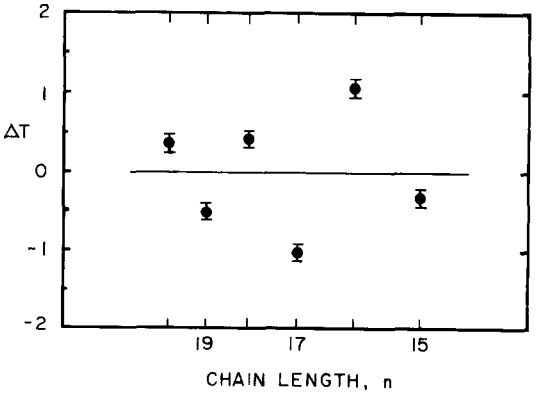

Fig. 4. The differences in the dilatometric transition temperatures T_{GF} from the straight line in Fig. 3 as a function of $1/(n-3.7)$.

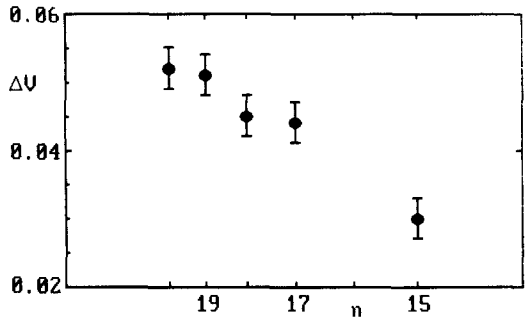

Fig. 5. Volume changes, ΔV_{GF}, in ml/g of lipid, at the G to F transition as a function of $1/(n-3.7)$. Selected values of n are shown.

have not been completely clarified [5]. The samples upon which the present dilatometry studies were performed were clearly not fully incubated and had transition temperatures T_{CG} of about 16.2°C when scanned very slowly and about 16.6°C when scanned at 2.5 K/h. Similarly, for 19i the calorimetric T_{CG} was 29.3°C (shown in Fig. 3), whereas the dilatometric T_{CG} for very slow scans on partially formed C phase lipid was only 26.9°C (given in Table I). Because of these long incubation time considerations, the values of ΔV_{CG} reported in Table I for 17i and 19i are likely to be only lower bounds for the true values.

The results reported in Table I for 21i were obtained by differential scanning calorimetry. Apparent disagreement for the value of T_{CG} from that of [2] was resolved by incubation studies illustrated in Fig. 6. For short incubation times, there is only one peak near 27°C, in agreement with [2]. When the incubation time is increased to 580 h at 1°C one can see a peak at 27°C, accompanied by a shoulder near 28.5°C, and a well separated peak at 34°C. It has previously been found in DPPC [9] that jumping to a higher incubation temperature after initial incubation at a low temperature speeds up the conversion to the final state. Fig. 6 shows the calorimetric results of two temperature jumps for 21i. The jump to 14°C for 71 h results in a substantial shoulder on the 27°C peak. The jump to 22°C for 133 h results in the 34°C peak becoming the largest one and resolution of the peak at 28.5°C. However, in the latter experiment the total enthalpy under all three peaks is less than for the very long incubation at 1°C; at first glance this would suggest that the enthalpy of transition is less for those bilayers of 21i which have converted to the ultimately stable form that melts at 34°C than for those bilayers that are in less stable forms. A more reasonable alternative is that nucleation only occurs at low temperatures, even though growth of an already nucleated phase occurs faster at higher temperatures, and by the assumption that not all the bilayers are nucleated in 36 h at 1°C.

The T_{CG} temperature reported in Table I for 18i was determined from heating scans by the following procedure. The GF transition was separately measured by cooling the system to 31°C, which is between T_{GF} and the lower transition temperature observed when cooling in Fig. 1, and starting the heating scan from there. It is found that the measured heating transition is then identical to the upper portion of the total CF transition.

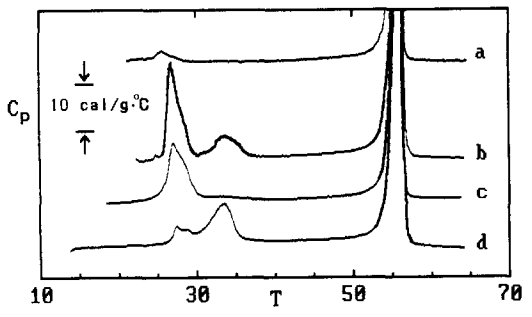

Fig. 6. Differential scanning calorimetry results for 21i with different histories of incubation: (a) 31 h at 1°C, (b) 580 h at 1°C, (c) 37 h at 1°C followed by 71 h at 14°C, and (d) 36 h at 1°C followed by 133 h at 22°C.

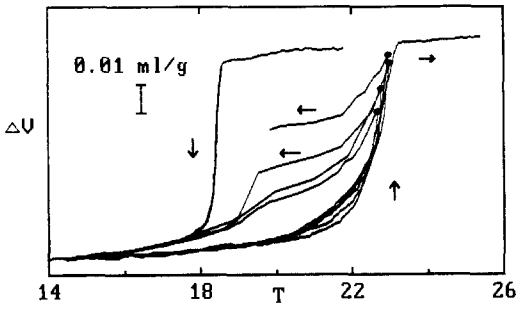

Fig. 7. Temperature-reversal dilatometric data for 16i. Shown are a complete heating scan (4.8 K/h) and a complete cooling scan (4.3 K/h) as well as several heating scans in which the temperature (°C) was reversed at the large black dots part way through the transition. Typical heating rates were 5 K/h followed by cooling rates after reversal of 0.1 K/h.

We next subtract the separately measured GF transition from the measured CF transition in Fig. 1 to obtain ΔV_{CG} and T_{CG} given in Table I. It may also be noted that the larger lowering of the CG transition temperature observed in cooling scans suggests that formation of the C phase is slow for 18i. However, it is much faster than in its odd n analogues, and it is also faster than in 20i, which needs about 5 h at 4°C to form its C phase completely.

The results for the temperature-reversal measurements are shown in Fig. 7 for 16i. For comparison, also shown are a complete heating scan followed by a complete cooling scan, both scans proceeding through the entire transition region (14 to 26°C). When the temperature is reversed before completion of the transition in the heating scan, the volume change due to the transition partially reverts, indicating that some of the F phase is resolidifying. Reversion is incomplete, however, until the temperature is lowered to near the temperature at which the transition occurs during a full cooling scan.

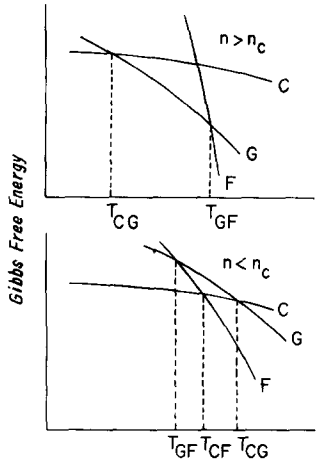

Fig. 8. Qualitative Gibbs function plots as a function of temperature for the three phases C, G and F for the isobranched phosphatidylcholines. Longer chain lengths with $n > n_c$ are represented at the top of the figure and shorter chain lengths with $n < n_c$ are represented at the bottom of the figure. The location where the C and G curves cross give the temperatures T_{CG}. T_{GF} and T_{CF} are found in a similar way from the respective curves.

Discussion

A. Phase transitions: equilibrium

An outstanding feature of this family of lipids is the change in the number of phase transitions that takes place as a function of chain length n. For the larger n values studied there are two distinct transitions at temperatures T_{CG} and T_{GF}. As n decreases the temperature difference $T_{GF} - T_{CG}$ decreases until, for the shorter n values studied, only one transition occurs from the C phase directly to the F phase. This may be comprehended in a simple way in the Gibbs diagrams in Fig. 8; such diagrams will be very useful in discussing a number of aspects of this family of lipids. Each phase, F, G and C is presumed to have a smooth Gibbs function G as a function of temperature, as shown in Fig. 8. At a particular temperature that phase with the lowest value of G is the stable phase and a first-order transition occurs as temperature is varied when the Gibbs function of the formerly stable phase is crossed by the Gibbs function of another phase, provided that equilibrium is achieved. As shown in the top of Fig. 8, for the higher n values studied the Gibbs curve for the C phase is crossed, as T is increased, by the Gibbs function for the G curve at T_{CG}. In turn the Gibbs function for the G curve is crossed by the Gibbs function for the F curve at T_{GF}. In contrast, for the smaller values of n studied, the Gibbs function for the G curve is not the minimum G function at any temperature. As shown in the lower part of Fig. 8, only C and F are stable phases with a transition temperature which we will call T_{CF}. The crossing of G with C and F will also be called T_{CG} and T_{GF} even though equilibrium phase transitions do not take place at these latter two temperatures.

The Gibbs function description in the preceding paragraph leads quite naturally to the inclusion of the chain length n as another variable, albeit n is a discrete and not a continuous variable. As n is varied, the graph of each Gibbs function for each phase varies, leading to different values of the T_{CG}, T_{GF} and T_{CF} temperatures. In this simple phenomenological picture the striking behavior of having two transitions at higher n values and only one transition at lower n values is simply due to a shift upward of the Gibbs function for the G curve relative to the Gibbs functions for the C and F curves. The value of n at

which the three crossover temperatures become equal, $T_{CG} = T_{GF} = T_{CF}$, will be called the confluent value, n_c. For values of n greater than n_c there are two equilibrium transitions, one at T_{CG} and one at T_{GF}. For values of n smaller than n_c there is only one equilibrium transition at T_{CF}. Clearly, n_c need not be an integer value but may be defined by interpolation of the realizable integer values. Also, the data show that, as with most hydrocarbon chain properties, there is an odd/even effect, so n_c is close to 15 for odd n and n_c is slightly smaller than 18 for even n.

The T_{GF} transition temperatures of these isobranched lecithins exhibit the expected trend with increasing chain length as shown in fig. 3. The fact that the intercept at $n = \infty$ is close to the melting temperature of polyethylene is expected because in the long chain limit the lipid hydrocarbon chains differ from polyethylene chains only by perturbations near the chain ends, by the isobranched methyl on the free end and by the headgroup on the other end. The sequences of straight-chain phosphatidylcholines [9] and the straight-chain cephalins [14] also extrapolate to the melting temperature of polyethylene (with $\delta = 3$) as do the long chain alkanes (with $\delta = -5$) [8].

B. Kinetics of the phase transitions

An odd/even effect is observed not only in the transition temperatures T_{GF} (see Fig. 4) but also in the hysteresis of the GF transition, as shown in Fig. 2, with the even n lipids requiring longer equilibration times to form the G phase. The much stronger odd/even variation in the T_{CG} transition temperatures has been reported earlier [2]. The present study also emphasizes that the incubation times necessary to form the C phase exhibit a strong odd/even effect with the odd n lipids requiring much longer incubation times. The contrast in the odd/even hysteresis effect for the T_{GF} transition, for which even n lipids require longer incubation times, to the odd/even hysteresis effect in the T_{CG} transition, for which the odd n lipids require longer incubation times, should not make one lose sight of the common property of a systematic slowing down of transition kinetics with increasing chain length despite the higher temperatures at which the transitions occur.

In an earlier study [2] it was proposed for the shorter-chain lipids, for which an F to C transition takes place upon cooling, that the transition proceeds via the G phase. Operationally, in the lower part of Fig. 8 this means that upon cooling from the F phase to temperatures between T_{CF} and T_{GF} the system becomes metastable and remains in the F phase until the temperature is lowered to below T_{GF}. As soon as the temperature is lowered below T_{GF} the G phase becomes stable with respect to the F phase and begins to form with measurably fast rates. However, at these temperatures the G phase is unstable with respect to the C phase and rapidly converts into it. If this is true, then the transition temperature upon cooling for the shorter-chain lipids that have only one transition, i.e., $n < n_c$, is the T_{GF} temperature in the Gibbs plot in the lower part of Fig. 8. The fact that the measured cooling transition temperatures for the shorter-chain lipids extrapolated so well from the T_{GF} temperatures for the longer-chain lipids as shown in Fig. 3 was one of several reasons for suggesting this hypothesis [2]. The hypothesis makes sense because nucleation of a well-ordered C phase straight from a fluid F phase could be kinetically slow. None of the results in this paper are contrary to this hypothesis.

The converse of the above hypothesis was also proposed [2], namely that whenever the shorter-chain lipids are melted from the C phase to the F phase, the transition again takes place via the G phase. In terms of the Gibbs diagram (Fig. 8) this would mean that upon heating from the C phase the system would proceed all the way to T_{CG} before the transition to F would take place. While logically possible, this converse hypothesis is considerably more radical than the cooling hypothesis because nucleation of a fluid phase from a solid, which is never completely free from imperfections such as dislocations, disclinations, grain boundaries, etc., is usually thought to be much easier. Therefore, we have tested this converse hypothesis in this paper using the temperature-reversal procedure.

The results in Fig. 7 show, indeed, that the converse hypothesis does not hold. Upon heating part way through the transition, part of the system has been converted into the F phase and part is still solid. Upon temperature reversal, Fig. 7 shows that some of the F phase converts back to solid.

This is inconsistent with the heating melting temperature occurring at T_{CG} in Fig. 8 because at that temperature the F phase is much more stable than either the C or G phases and would not revert back upon cooling. If the converse hypothesis were true, then the F phase would either continue to grow or remain constant in size; this would require the volume to continue to increase upon temperature reversal in Fig. 7 or to decrease only slightly as in the pure C and F phases. The fact that the decrease is much larger than occurs for pure single phase behavior is positive proof that the transition temperature upon heating is T_{CF}.

If partial melting of the sample resulted in complete melting of a fraction of the liposomes and no melting of the remaining liposomes, then in the temperature-reversal experiments the volume upon cooling would decrease only slightly, typical of single phase behavior. This did not happen, but it could have if the time had been sufficiently long for the system to anneal before beginning the temperature reversal. What must instead happen is that many, though not all subunits (e.g. liposomes or bilayers within liposomes or even smaller effective subunits), must consist of domains of F phase and C phase. In such subunits the stable C phase grows at the expense of the unstable F phase upon temperature reversal.

We now present a simple model that mimics the behavior in Fig. 7 and that gives a formula to which those results can be compared. Let us assume that all the subunits are of similar size and that there are a total of N of them, and in each subunit there are M domains which will be either in the F phase or in the C phase. In the model, if all M domains in a subunit are in the F phase then this subunit does not return to the C phase upon temperature reversal, but if even one domain in a subunit is in the C phase, then the subunit will completely return to the C phase. We define X to be the ratio of the amount converted to the F phase at the maximum temperature achieved before temperature reversal to the total amount and we define Y to be the ratio of the amount that does not solidify upon temperature reversal to the total amount. Assuming that, upon heating through the transition, each domain is in the F phase independently of other domains, then X is just the probability of any domain being in the F

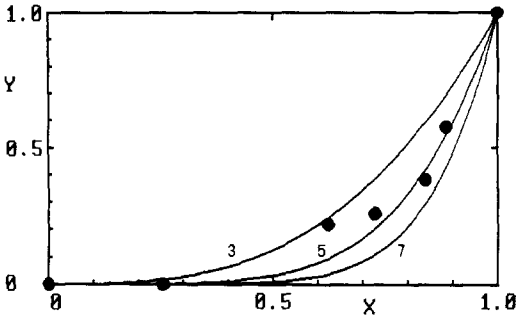

Fig. 9. Data points give the fraction of 16i melted at the highest temperature before temperature reversal on the x axis and the fraction that remains melted upon temperature reversal on the y axis. The three solid curves for $M = 3$, 5 and 7 give the result of a simple two-state domain theory described in the text.

phase. The fraction Y that does not revert to the solid phase upon temperature reversal is just the probability that all M domains in any subunit are in the F phase; this is just X^M. Fig. 9 shows the Y vs. X plot for the experimental results and the model results for $M = 3$, 5 and 7. Not surprisingly, in view of the oversimplified theory, which is similar to two-state melting theory, quantitative agreement between experiment and theory is not perfect for any value of M. Nevertheless, the fact that both theory and experiment exhibits the same qualitative trends in Fig. 9 supports the domain picture for the temperature-reversal experiments.

C. Bilayer structure

In this subsection the recent X-ray structural results of Church et al. [5] will be combined with our apparent specific volume measurements to yield more information than can be obtained from either technique alone. Various structural parameters that describe the bilayers in the C and G phases of 17i and 20i are listed in Table II. The first row lists the low angle X-ray spacing D (in Å) between successive layers in the multilamellar arrays. The second row is the area A_H (in Å^2) which is perpendicular to D; A_H has been determined by X-ray diffraction only for the C phases. The third row is the volume $V_X = A_H D/2$ which is the average volume (in Å^3) of one lipid and its associated waters. In the fourth row our absolute apparent specific volumes, v_M (in ml/g),

TABLE II

STRUCTURAL PARAMETERS

See text for definitions, data and calculations.

	17i		20i	
	C (−4 °C)	G (21 °C)	C (0 °C)	G (47 °C)
1 D (Å)	58.0	61.0	61.8	67.2
2 A_H (Å^2)	39.8	42.3	47.7	43.0
3 V_X (Å^3)	1154	1290	1474	1445
4 v_M (ml/g)	0.909	0.949	0.917	0.981
5 n_W	0.1	3.0	6.2	2.2
6 V_L (Å^3)	1150	1201	1288	1378
7 V_{HC} (Å^3)	809	860	947	1037
8 V_H (Å^3)	341	341	341	341
9 θ	0	0	35	0

are given. The fifth row lists the average number of waters per lipid n_W.

By assuming that the volumes of various parts of the molecules, e.g., the headgroups and the methylene groups, are numerically equal to the values that have been derived for other phases for which no unit cell volumes are available [9,15], Church et al. [5] estimated the number, n_W, of water molecules per lipid molecule in the C phase to be 0–2 for 17i and 7–8 for 20i. The very small value of n_W for 17i is quite surprising. Our measured values of absolute apparent specific volume, v_M, determine n_W directly from the unit cell volumes from the formula

$$v_M = 0.6023\, V_X/(m_L + n_W m_W) \quad (1)$$

where 0.6023 converts from volumes in Å^3/molecule to ml/mole, m_L is the molecular weight of the lipid and m_W is the molecular weight of the $^2H_2O/H_2O$ mixture at which the lipid neither sinks nor floats. Using Eqn. 1, n_W is found to be 0.1 for 17i and 6.2 for 20i in the C phase. The fact that these values are in reasonable agreement with those obtained in Ref. 5 indicates that the assumptions for the volumes of the various groups comprising the lipid were fairly good.

Row 6 in Table II lists V_L which is the volume per lipid molecule determined by

$$V_L = v_M m_L/0.6023 \quad (2)$$

This equation assumes that the volume of the water molecules, V_W, between the bilayers equals the volume of bulk water, V_S. This is essentially a convention since there is no rigorous way to separate the volume of the interlamellar water from the headgroup volume.

It is of interest to divide V_L into a part due to the hydrocarbon chains, V_{HC}, and a part due to the headgroups, V_H, which are listed in rows 7 and 8, respectively, in Table II. Finally, in row 9 is listed the angle of tilt θ of the chains with respect to the bilayer normal. For all-*trans* chains

$$V_{HC} = D_{HC} A_H \cos\theta \quad (3)$$

where D_{HC} is the length of the chain. By convention the carbonyl carbon is included in the headgroup and the terminal methyl has twice the length of a methylene group [15], so for iso-branched lipid ni, $D_{HC} = (ni - 1) \times 1.27$ Å. Applying these relations to 17i in the C phase for which Church et al. [5] have stated that θ is zero yields $V_H = 341$ Å^3. This is essentially the same as the value 344 Å^3 deduced in Ref. 9 for DPPC in the G phase. The excellent agreement for the volume of the PC head group, ranging over PCs in phases with both large and small values of n_W, suggests that we may set $V_H = 341$ Å^3 for the other entries in Table II for which either A_H has not been measured (the G phases) or for which θ is only indirectly determined (C phase of 20i).

To obtain the values in Table II for the G phase of 17i we first calculate $V_{HC} = V_L - V_H$. Assuming all-*trans* chains and taking $\theta = 0$ from Church et al. [5], Eqn. 3 gives A_H from which V_X and then n_W are calculated. Exactly the same procedure was followed to obtain the remaining values for the G phase of 20i. For the C phase of 20i we could use the value of $\theta = 33°$ given in Ref. 5. However, that value of θ required the assumption that the cross sectional area per chain is 20 Å^2, so we prefer to calculate V_{HC} using V_H and V_L and then use Eqn. 3 with the all-*trans* value of D_{HC} and the measured A_H to obtain $\theta = 35°$.

Discussion of structural parameters for the fluid phase requires a somewhat different approach from the one above for several reasons. First, the assumption needed to calculate D_{HC}, namely that the chains are all-*trans*, is clearly a poor one. Also, there are no structural data for A_H and θ is not well defined. Instead, we employ the assumption

that the volumes of the methylene groups (and the methyl groups, respectively) will be the same at the same temperature in the fluid phase for all the ni lipids. The reasonableness of this assumption was discovered for straight-chain PCs in Ref. 9, although a different assumption was made in analyzing the data in Ref. 9, namely, following Ref. 15, that the terminal methyl has a volume twice that of a methylene. (Our assumptions are clearly different from the assumption used in Ref. 16. A detailed discussion of the relative plausibility of the two assumptions will be published later.) For the isobranched lipids it is clearly necessary to add in some additional volume for the isobranched methyl. In Fig. 10 is shown the volume per methylene, V_{CH_2}, calculated in the following way. Our measured v_M was used to calculate V_L from which $V_H = 341$ Å^3 was subtracted to obtain V_{HC} which was then divided by $(ni + \gamma)$ to obtain V_{CH_2}. The value of γ that minimized the differences in the V_{CH_2} for the different ni lipids at the same temperature in the F phase only was $\gamma = 0.2$. Note that the number of methylenes is $ni - 3$ (where the penultimate CH group is counted as a methylene), so $\gamma = 0.2$ corresponds to the two methyl groups on each chain occupying a volume of 3.2 V_{CH_2}. By our assumption that V_{CH_2} ought to be the same for the different ni lipids at the same temperature in the F phase, this value of γ determines the volume that the methyls should occupy. The corresponding V_{CH_2} volumes computed for $\gamma = 0.2$ are shown in Fig. 10.

Fig. 10 also shows V_{CH_2} for the C and G phases. These volumes were calculated assuming

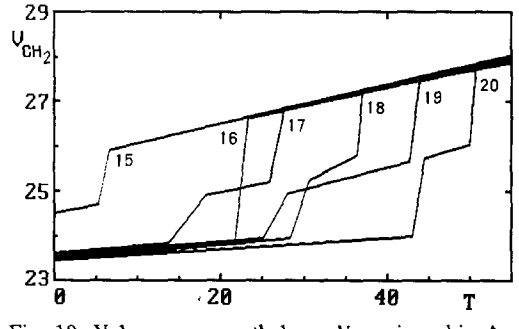

Fig. 10. Volumes per methylene, V_{CH_2}, in cubic Angstroms versus temperature (°C) for the ni isobranched lipids. The volumes of the pair of methyls on each chain end was 3.2 V_{CH_2} in the calculations.

that the volume of the methyls remains 3.2 V_{CH_2}. Due to severe packing constraints of the isobranched methyl in the more ordered phases, this makes these calculations at best a first approximation. It is tempting to compare these V_{CH_2} values with the product (1.27 Å)$(A_H/2)(\cos\theta)$ that can be computed from Table II because this latter product equals V_{CH_2} for straight-chain lipids. However, the values are not expected to be equal because this latter product is just a 1.27 Å high section of a parallelepiped whose total volume equals the volume of the chains and whose length D_{HC} equals the length of a straight-chain with no isobranch. Therefore, the fact that the product is larger than V_{CH_2} given in Fig. 10 is appropriate since the calculation of V_{CH_2} gives the isobranched methyl some volume, thereby decreasing V_{CH_2}. However, this difference calls attention to the thorny problem of the detailed packing of the isobranched methyls in chain ordered phases. Until we understand that packing better it seems inappropriate to attempt to calculate energy changes due to the cohesive van der Waals energies or to try to balance the energy equation in a similar way as for straight-chains [9,17].

The assumption that the methylene volumes should be the same at the same temperature in fluid, chain-melted phases should if true, also apply to different classes of lipids and to alkanes. For straight-chain PCs V_{CH_2} equals 27.7 Å^3 at 50°C [9], in good agreement with the V_{CH_2} for the isobranched PCs in Fig. 10. Some results are also available for PEs [14]. For DLPE V_{CH2} is about 27.8 Å^3. The result for V_{CH_2} for DMPE is too low in that paper because the measured ΔV at the chain melting transition is too low as shown by Ref. 12. Correcting for this yields a value of V_{CH_2} of about 27.6 for DMPE. From appendix 1b in Ref. 16 one obtains 27.6 Å^3 for the alkanes. Therefore, it appears that this assumption is consistent across different classes of lipids.

Acknowledgements

We wish to acknowledge Dr. S. Tristram-Nagle for helpful discussions. This research was supported by Grant GM 21128 from the U.S. National Institutes of Health (J.F.N.) and by grants from the Medical Research Council of Canada

and the Alberta Heritage Foundation for Medical Research (R.N.M.).

References

1 Silvius, J.R. (1982) Lipid Protein Interactions (Jost, P.C. and Griffith, O.H., eds.), Vol. 2, p. 239, Wiley, New York
2 Lewis, R.N.A.H. and McElhaney, R.N. (1985) Biochemistry 24, 2431–2439
3 Silvius, J.R. and McElhaney, R.N. (1979) Chem. Phys. Lipids 24, 287–296
4 Mantsch, H.H., Madec, C., Lewis, R.N.A.H. and McElhaney, R.N. (1985) Biochemistry 24, 2440–2446
5 Church, S.E., Griffiths, D.G., Lewis, R.N.A.H., McElhaney, R.N. and Wickman, H.H. (1986) Biophys. J. 49, 597–605
6 Kannenberg, E., Blume, A., McElhaney, R.N. and Poralla, K. (1983) Biochim. Biophys. Acta 733, 111–116
7 Suzuki, A. and Cadenhead, D.A. (1985) Chem. Phys. Lipids 37, 69–82
8 Broadhurst, M.G. (1962) J. Res. Natl. Bureau Standards 66A, 241–249
9 Nagle, J.F. and Wilkinson, D.A. (1978) Biophys. J. 23, 159–175
10 Wilkinson, D.A. and Nagle, J.F. (1979) Biochemistry 18, 4244–4249
11 Nagle, J.F. and Wilkinson, D.A. (1982) Biochemistry 16, 3817–3821
12 Wilkinson, D.A. and Nagle, J.F. (1984) Biochemistry 23, 1538–1541
13 Wilkinson, D.A. and Nagle, J.F. (1978) Anal. Biochem. 84, 263–271
14 Wilkinson, D.A. and Nagle, J.F. (1981) Biochemistry 20, 187–192
15 Tardieu, A., Luzzati, V. and Reman, F.C. (1973) J. Mol. Biol. 75, 711–733
16 Small, D.M. (1986) The Physical Chemistry of Lipids, Plenum Press, New York
17 Nagle, J.F. (1980) Annu. Rev. Phys. Chem. 31, 157–195