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Density measurements through the gel and lamellar phase transitions of di-tetradecanoyl- and di-hexadecanoyl-phosphatidylcholines: observation of slow relaxation processes and mechanisms of phase transitions

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Density measurements on aqueous dispersions of C14:0/C14:0 and C16:0/C16:0 phosphatidylcholines have been carried out to give information on the mechanism of the phase transitions [main transition: lamellar phase (L_α) to gel ($P_{\beta'}$); pre-transition: gel ($P_{\beta'}$) to second gel phase ($L_{\beta'}$)]. Quite remarkably, for both systems we observe that repeated temperature cycles give different measurements, with a slow relaxation to apparent equilibrium, at least for some of the phases. We also observe that there are marked reductions in lipid density just above the main transition that indicate the spontaneous formation of 'lipid patches'. The density measurements allow the calculation of the relative contributions to the phase transitions from changes in van der Waals energy (ΔU^{vdW}) and chain configurations (ΔU^{Rot}). Both contribute to the main transition whilst only changes in the van der Waals energy are involved in the pre-transition.

1. Introduction

Phospholipid bilayers constitute one of the primary structural elements in biological membranes and hence their physical properties are of considerable experimental and theoretical interest. Membrane physical properties are presumed to influence processes such as intracellular interactions, transmembrane transport and signalling, changes in cell surface morphology, and the functioning of membrane-associated proteins [1]. Of particular interest at present is the recent discovery of lipid 'rafts'. These 'rafts' form distinct patches within the bilayer, and have very different physical properties (e.g. solubility in surfactant micelles) [2–4] from the rest of the membrane lipids. Some insight into the nature of biological membranes can be gained by studying synthetic bilayers consisting of a single type of phospholipid, mixtures of phospholipids or phospholipids with proteins or peptides. Such systems are sufficiently simple to permit a systematic analysis of their properties, yet complex enough to retain certain aspects of the essential properties associated with their biological counterparts. It is of little surprise then, that lipid bilayers (in water) have been much studied as the prototypical membrane. In addition to their obvious biological importance, synthetic membranes are also of intrinsic physical interest as soft material systems that self-assemble, and because they can act as surfactants, which themselves have very broad applications in products such as conditioners, creams and emulsions [5].

Lecithins (1,2-dialkanoyl-*sn*-glycero-3-phosphatidylcholines) are probably the most widely studied lipids, with the 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (C14:0/C14:0, DMPC) and 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (C16:0/C16:0, DPPC) derivatives being the most popular. Much has been documented about the phase changes of lecithin/water mixtures as a function of lipid chain length, so only a brief summary will be given of established behaviour. At high temperatures a lamellar phase (L_α), figure 1 (*a*), is formed over some composition range, and on cooling is transformed into a gel phase ($L_{\beta'}$, $P_{\beta'}$), figures 1 (*b*) and 1 (*d*).

Within the gel phase the lipid chains are in a semi-rigid state, having much reduced conformational freedom compared with the liquid-like disorder of the lamellar phase. There are several different structural

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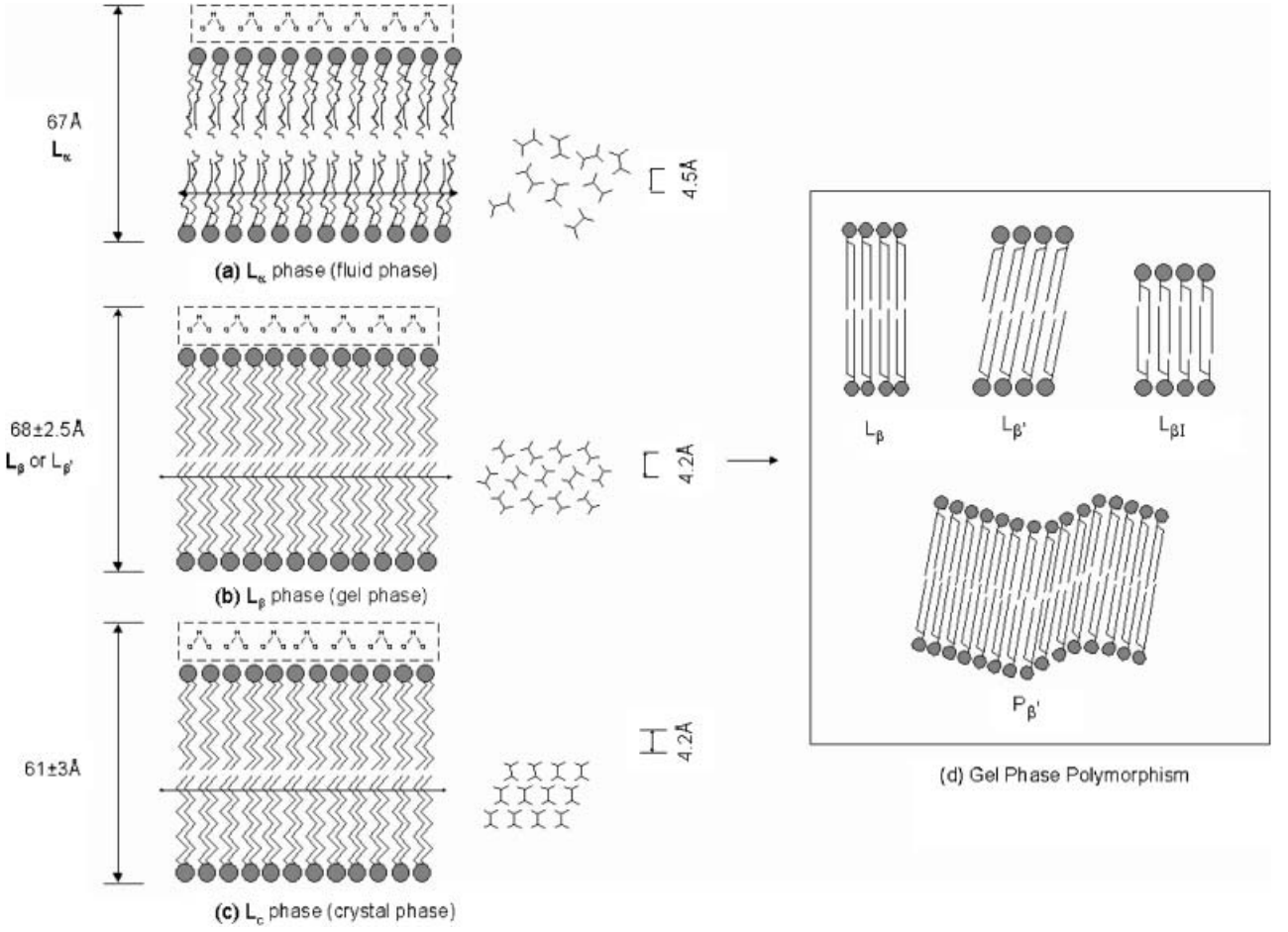

Figure 1. Lipid bilayer polymorphism: (*a*) L_α lamellar phase, (*b*) L_β normal gel phase, (*c*) L_c crystalline phase, (*d*) various gel phase structures including L_β normal gel phase, $L_{\beta'}$ tilted gel phase, $P_{\beta'}$ ripple gel phase, $L_{\beta I}$ interdigitated gel phase. All dimensions are for DPPC [1].

arrangements of the alkyl chains within the gel layers, figure 1 (*b*). After the formation of the initial gel phase on cooling from the lamellar phase (at the *main transition*, T_M), there is a second transition (the '*pretransition*', T_P) to a second gel phase, typically at 10–20°C below the lamellar/gel transition. It might be thought that, with the vast array of publications on these materials, there was little new to discover. However, there are numerous aspects of the gel phases that remain unexplained. For example, in the ripple phase ($P_{\beta'}$), what is the exact molecular arrangement? Clearly, not all the molecules have an identical configuration. Even in the case of the simple gel structure with vertical chains, the 1- and 2-positions have different chain configurations close to the head group; hence there must be differences between the two chains in the packing of the methyl group region. Moreover, the double chain structure makes it impossible for the molecules to pack into uni-axial layers (as is usually assumed). A major unknown is the nature of the gel/second gel phase transition; the latter is often considered to be a crystal, but a detailed examination of the phase transition enthalpies suggests that more often than not it is a second type of gel phase.

Recently we began some accurate measurements of densities for a range of lecithin/water systems using the often overlooked vibrational density meter technique. This paper reports our initial observations that show remarkable slow relaxation processes and significant temperature hysteresis, not previously reported. Our aim was to investigate how the thermodynamic and structural properties change as the component lipids are varied. It has long been realized that the volume of the lecithin changes with temperature and phase, however relatively little has been published regarding the intra-bilayer energy changes involved. A notable exception is the work of Nagle, who has conducted very significant investigative work in this area. To calculate the internal

energy changes involved with the phase transition, the heat of transition was written as

$$\Delta H = \Sigma \Delta U_i + p\Delta V \quad (1)$$

where ΔU and ΔV are the changes in the internal energy and volume, and p is the pressure at the transition temperature. For complex molecules such as phosphatidylcholines there are many possible different internal energies. However, the two largest contributors are believed to be: (i) the energy associated with a change in the number of *gauche* rotamers on formation of the new phase (ΔU^{Rot}), and (ii) the change in van der Waals interactions between the hydrocarbon tails (ΔU^{vdW}). Assuming that the volume of the lipid headgroup is constant, Nagle and Wilkinson were able to estimate the change in volume per CH_2 group (V_{CH_2}) with temperature and extracted the van der Waals and rotameric interaction energies [6].

In this paper two symmetrical lecithins (C14:0/C14:0 and C16:0/C16:0) are studied. We have measured densities for dispersions (5 wt%) of the lecithins in water over a sequence of heating/cooling runs. A significant hysteresis in the gel phase densities is observed, with a slow relaxation to a constant value. Also, we observe relatively large deviations from the expected values for the lamellar phase density just above the lamellar/gel transition. This is a significant pretransitional behaviour that could have implications for the structure of biological membranes.

2. Experimental methods

2.1. *Materials*

1,2-Dimyristoyl-*sn*-glycero-3-phosphatisylcholine (DMPC, M_w=677.9 g mol^{-1}) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatisylcholine (DPPC, M_w=734.05 g mol^{-1}) were commercial materials (>99.9% purity) purchased from Avanti Polar Lipids (Alabaster, AL, USA) in the lyophilized form, and used without further purification. The water for preparation of the dispersions was distilled and deionized. All lecithin powders were stored at −18°C [7].

2.2. *Sample preparation and measurements*

Lecithin dispersions were prepared using a technique similar to that used by Nagle and Wilkinson [6] for aqueous lipid samples. Care was taken to limit the exposure of the powder and aqueous lecithin to air, thus minimizing degradation. The 3 g (5 wt% lecithin) aqueous sample was vigorously stirred for 24 h in a sealed vial above the main phase transition temperature (typically 55°C) before it was syringed into the sample capillary tube of the density meter, quickly cooled to 5°C, and held at that temperature for 24 h. This temperature incubation has been reported to ensure equilibration between the $L_{\beta'}$ and $P_{\beta'}$ phases [8] and gives a constant starting point for all the lipid samples. Density measurements were made with a DMA 5000, Anton Paar density meter (resolution 1×10^{-6} g cm^{-3}) at a heating and cooling rate of *c.* 7°C h^{-1}. [The DMA 5000 density meter is calibrated annually by UKAS accredited H&D Fitzgerald Ltd., whose density measurements are traceable to national standards held by the National Physical Laboratory.] The sample experienced heating from 5 to 55°C followed by an immediate cooling back to 5°C. Density measurements were recorded at increments of 0.5°C. The same procedure was used for both of the lecithins examined.

3. Theory

The following section comprises a basic description of the analysis used in determining the energies associated with the phase transitions of the lipid systems. First we describe the method used to estimate the volume occupied by the lipid alkyl chains as a function of temperature. Then these values are employed to calculate the energies associated with the phase transitions.

3.1. *Estimation of lipid volume*

The molecular volume $V_{\mathrm{Dispersion}}$ of the overall dispersion can be divided into separate contributions from the lipid and water:

$$V_{\mathrm{Dispersion}} = x_w \overline{V}_w + x_L \overline{V}_L \quad (2)$$

where $\overline{V}_w$ and $\overline{V}_L$ are the partial molar volumes of the water and lipid respectively, and x_w and x_L are the mass fractions of the water and lipid, respectively. Making the assumption that the partial molar volume of water in the dispersion is equal to the molar volume of pure water V_w (which is dependent on temperature) allows the estimation of the volume occupied by a lipid molecule from measurements of the overall dispersion density.

The volume per lipid molecule V_L can be divided (see figure 2) into a contribution from the headgroup V_{head}, which also includes the glycerol region, and a contribution from the two tail groups $2V_{\mathrm{tail}}$:

$$V_L = V_{\mathrm{head}} + 2V_{\mathrm{tail}}. \quad (3)$$

It is often assumed that the headgroup volume is constant as a function of temperature for a specific lecithin [9]. This assumption is probably invalid for a comparison of the gel and lamellar phases, where the hydration of the head group is likely to change. It is

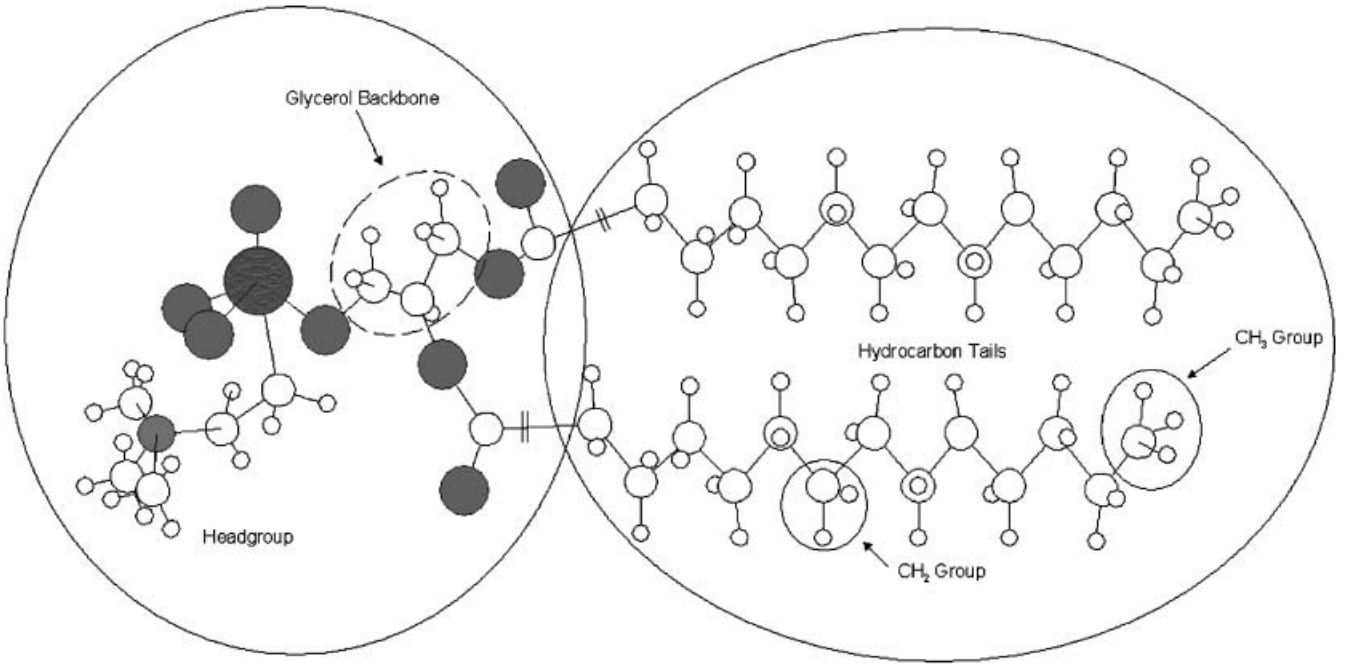

Figure 2. Structure of DMPC lipid. Indicated are V_{CH_2}, V_{CH_3}, the headgroup, glycerol and the two hydrocarbon tails.

more valid for the two gel phases, where the hydration of the headgroup is lower. Even in the first case, the error is likely to be only *c*. 10 Å^3 (based on a 5% volume change for seven bound water molecules per lipid), and will be almost the same for both lipids studied here. We have taken the headgroup volume (V_{head}), to be 319 Å^3 [9] for both lipids.

The volume of a tail (V_{tail}) can be expressed as the sum of the volumes of CH_2 and CH_3 groups within each tail:

$$V_{tail} = (n-2)V_{CH_2} + V_{CH_3} \quad (4)$$

where n is the length of the carbon chain for each tail.

3.2. *Calculating the volumes of CH_2 and CH_3 groups*

We assume that the hydrocarbon chain volume in the lamellar (L_α) phase is the same as that of a liquid alkane. It can also be argued that over a short temperature range (approximately 60°C) the volume change per alkane molecule is linear with temperature. Thus by fitting a straight line through alkane volume data, the volume change per CH_2 and CH_3 group can be estimated, using equations (5) and (6) and combined in equation (7):

$$V_{CH_2} = a + bT \quad (5)$$

$$V_{CH_3} = cV_{CH_2} = c(a+bT) \quad (6)$$

$$V_{alkane} = (n-2)(a+bT) + 2(c)(a+bT) \quad (7)$$

where a (18.5013), b (0.0307) and c (1.9000) are constants. Using this technique on straight chain alkanes the volume ratio of CH_2 to CH_3, c, was optimized to 1.90 ($V_{CH_3} = 1.9V_{CH_2}$), which is in close agreement with the value given by Nagle and Tristram–Nagle of 1.93 [9]; see figure 3. The optimization procedure is essentially the minimization of the error between the calculated value of V_{alkane}, equation (7) and previous experimental values [10, 11].

Recent published results have V_{CH_2} at 28.4 ± 0.4 Å^3 and V_{CH_3} at 53.9 ± 0.8 Å^3 in the L_α phase [9] (ignoring temperature dependence). The paper does not comment for which specific lecithin these volumes are, but they compare well to our calculated values of $V_{CH_2} = 28.4$ Å^3 and $V_{CH_3} = 53.9$ Å^3 in the L_α phase presented here. The technique generated results with an error of *c*. ± 0.5%.

3.3. *Estimation of the energies*

As in Nagle and Wilkinson [6], we use the relationship described in equation (1) to estimate the internal energy of the lecithin systems. The volume changes associated with the phase transitions are very small (approximately 10 Å^3 for the pretransition and 40–50 Å^3 for the main transition), so at atmospheric pressure the hydrostatic work term $p\Delta V$ equals approximately 5×10^{-5} kcal mol^{-1}. Therefore $p\Delta V$ is negligible in comparison to, e.g. $\Delta H \cong 9.1$ kcal mol^{-1}

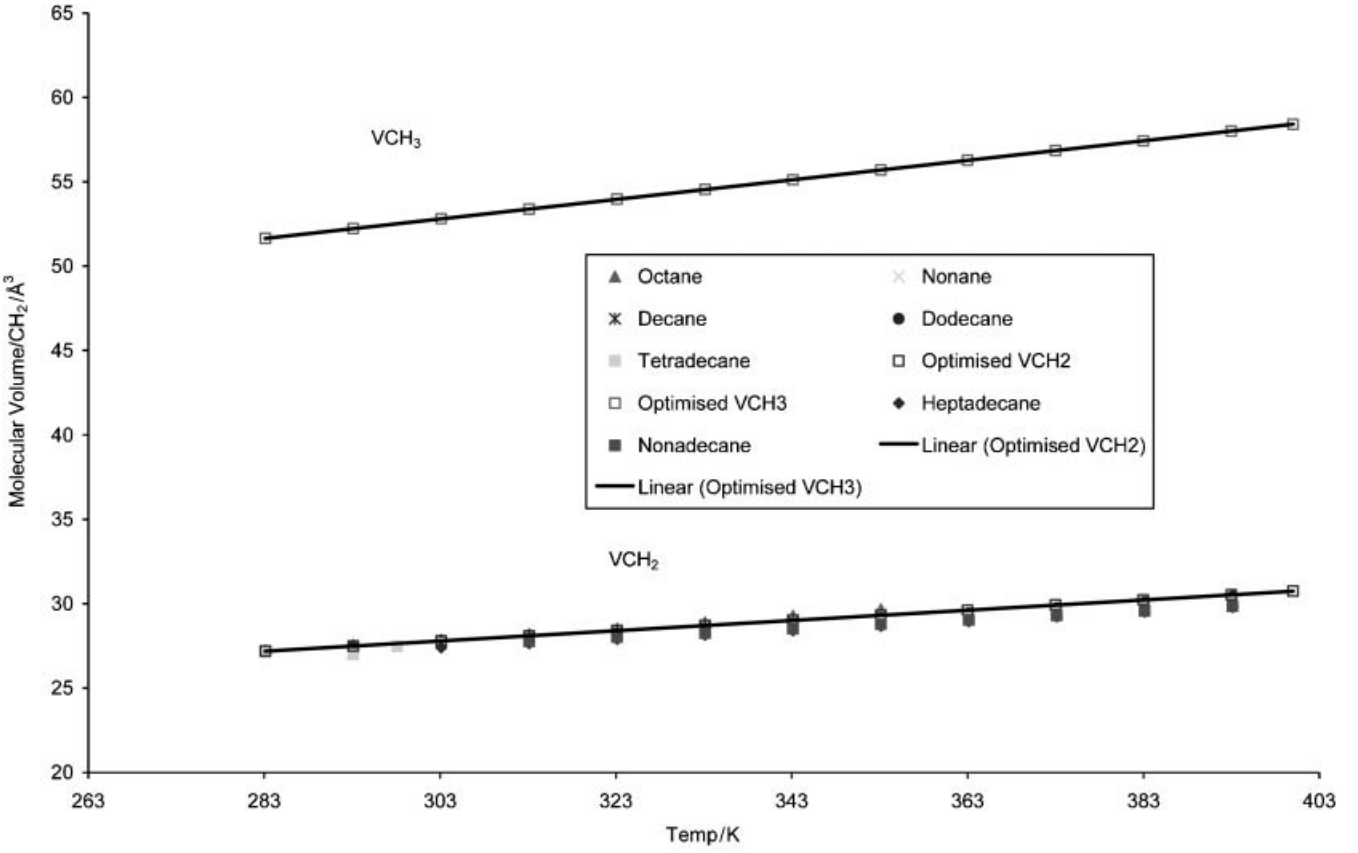

Figure 3. Volumes of CH_2 and CH_3 vs temperature. The trend lines represent optimized values to the fitting of equations (6) and (7). Data from [10, 11].

for DPPC [12] at the main transition. Thus it can be assumed that,

$$\Delta H = \sum \Delta U_i = \Delta U^{\mathrm{vdW}} + \Delta U^{\mathrm{Rot}}_{t/g} + \Delta U^{\mathrm{other}} \qquad (8)$$

where the internal energy comprises the van der Waals (ΔU^{vdW}), rotameric $\left(\Delta U^{\mathrm{Rot}}_{\mathrm{t/g}}\right)$ and all other energy ($\Delta U^{\mathrm{other}}$) respectively.

3.4. *Van der Waals interaction energy (ΔU^{vdW})*

To calculate the van der Waals energy, we make the assumption that the dispersion interaction energy between separate tail groups depends only on the local density of tail groups (which is inversely related to the molecular volume). We assume that the dispersion energy associated with a lipid CH_2 group in the three phases ($L_{\beta'}$, $P_{\beta'}$, and L_α) is the same as that of a CH_2 group in a linear alkane in the liquid phase at the same molecular volume.

The volume dependence of the dispersion energy of liquid alkanes can be quantitatively determined from heat of vaporization data. The dispersion energy ΔU^{vdW} is essentially the energy required to remove one alkane molecule from the liquid phase into the vapour phase (i.e. the heat of vaporization (ΔH^{vap}), minus the work of expansion ($p\Delta V^{\mathrm{vap}}$):

$$\Delta U^{\mathrm{vdW}} = \Delta H^{\mathrm{vap}} - p\Delta V^{\mathrm{vap}}. \qquad (9)$$

The alkanes in their vapour phase are assumed to behave as an ideal gas. This is valid provided the alkanes are below their critical temperature. As the lowest critical temperature of any of the alkanes used in the calculation is 568.7 K (for octane) then the assumption of the ideal gas law is justified. Therefore, the work of expansion $p\Delta V^{\mathrm{vap}}$ in equation (9) can be replaced by RT. Heats of vaporization data were used to calculate a value for ΔU^{vdW} for several linear alkanes using equation (9); the results are presented in figure 4.

The total van der Waals energy (ΔU^{vdW}) can be divided into a sum of contributions from all the CH_2 groups $\left(\Delta U^{\mathrm{vdW}}_{\mathrm{CH_2}}\right)$ and the two CH_3 groups $\left(\Delta U^{\mathrm{vdW}}_{\mathrm{CH_3}}\right)$ in the alkane:

$$\Delta U^{\mathrm{vdW}} = (n-2)\Delta U^{\mathrm{vdW}}_{\mathrm{CH_2}} + 2\Delta U^{\mathrm{vdW}}_{\mathrm{CH_3}}. \qquad (10)$$

To a first approximation, we assume that the differences between the interaction energies of CH_2 and CH_3 are

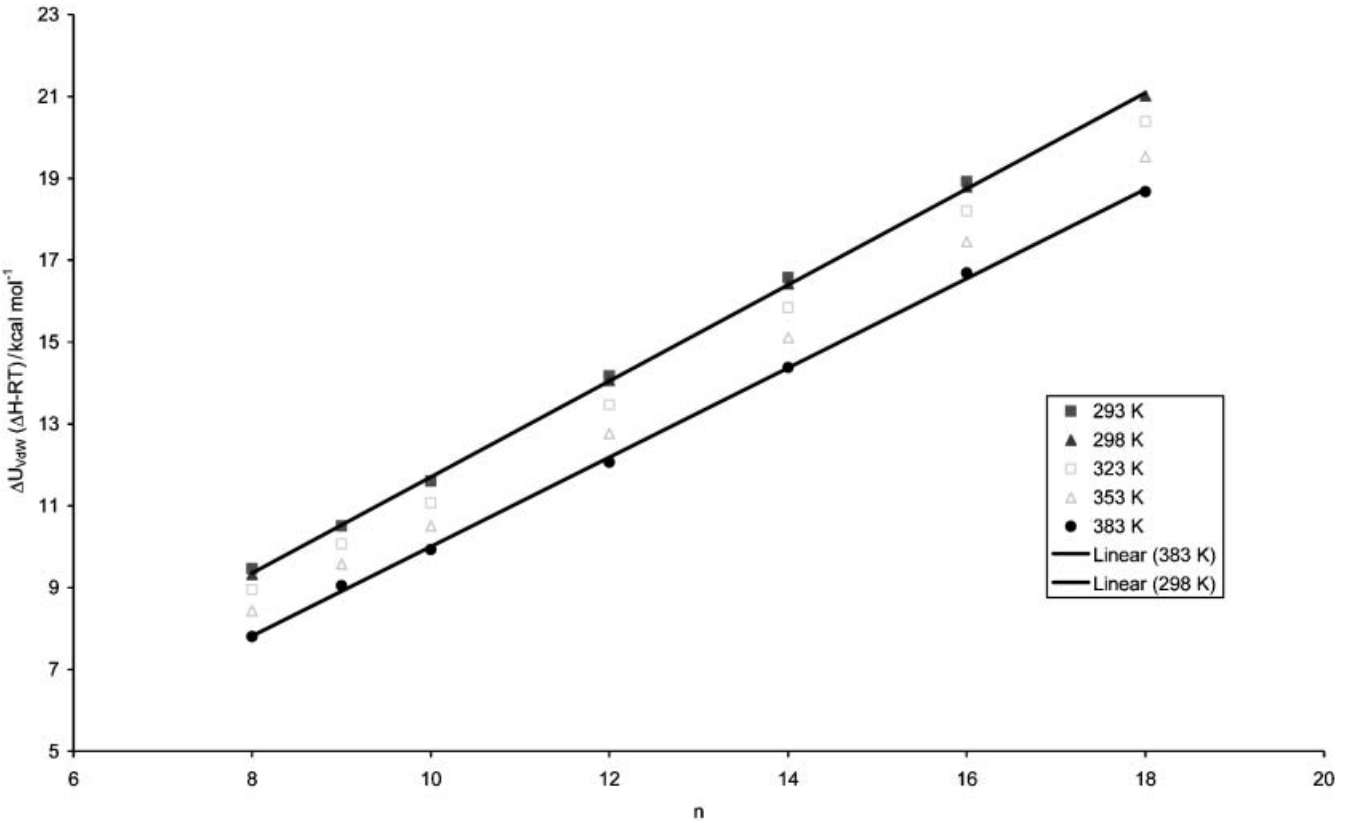

Figure 4. Change in experimental ΔU^{vdW} with n (number of carbon atoms). Data from [13].

negligible; then $\Delta U^{vdW}_{CH_3} = \Delta U^{vdW}_{CH_2}$. Values of $\Delta U^{vdW}_{CH_2}$ were obtained from the data in figure 4 using equation (10). The final values of $\Delta U^{vdW}_{CH_2}$ can be seen to vary linearly with temperature, see figure 5. In order to determine the volumetric dependence of the van der Waals energy, we plot the variation of $\Delta U^{vdW}_{CH_2}$ with respect to the volume occupied by a CH_2 group (V_{CH_2}) at the same temperature. This relationship is plotted in figure 6.

The second step uses the linear relationship (shown in figure 6) between U^{vdW} and V_{CH_2} to allow the calculation of the van der Waals energy change for any change in volume (ΔV_{CH_2}) over a transition. Now the total van der Waals interaction energy for the tailgroups can be expressed as,

$$\Delta U^{vdWtotal} = \Delta U^{vdW}_{CH_2}.n_{CH_2} + \Delta U^{vdW}_{CH_3}.n_{CH_3} \qquad (11)$$

and introducing the change in volume per CH_2 group (ΔV_{CH_2}) we get,

$$\Delta U^{vdW}\left(\frac{\Delta V_{CH_2}}{\Delta V_{CH_2}}\right) = \left(\frac{\Delta U^{vdW}_{CH_2}}{\Delta V_{CH_2}}\right) n_{CH_2}\Delta V_{CH_2} + \left(\frac{\Delta U^{vdW}_{CH_3}}{\Delta V_{CH_2}}\right) n_{CH_3}\Delta V_{CH_2} \qquad (12)$$

which simplifies to

$$\Delta U^{vdW} = \left(\frac{\Delta U_{CH_2/CH_3}}{\Delta V_{CH_2}}\right)(n_{CH_2} + n_{CH_3})\Delta V_{CH_2} \qquad (13)$$

where $\Delta U_{CH_2/CH_3}$ is the internal energy associated with either a CH_3 or CH_2 fragment. Therefore if ΔV_{CH_2} is known, the value $\Delta U_{CH_2/CH_3}$ can be estimated from figure 6. Thus it is possible to estimate ΔU^{vdW} using equation (13) if n is known, as $n_{CH_2} = (n-2)$ and $n_{CH_3} = 2$ for straight chain alkanes.

3.5. *Theory and calculation of rotameric energy*

Calculations of the rotameric energy are based on the rotational isomeric state approximation [14], where the dihedral angles of the tail groups are treated as occurring in either the *trans* (t), *gauche*$^+$ (g^+) or *gauche*$^-$ (g^-) states, which are located at the minima of the rotational potential, see figure 7. The energy difference between the *trans* and *gauche* states is approximately 0.5 kcal mol^{-1} [6]. Ordinarily, only the *trans* conformation is found in the crystalline phase. On gradual heating the probability of having either of the *gauche* conformations increases until after the gel/lamellar transition where it is believed that all of the conformations can exist, as in the free liquid phase [9, 14].

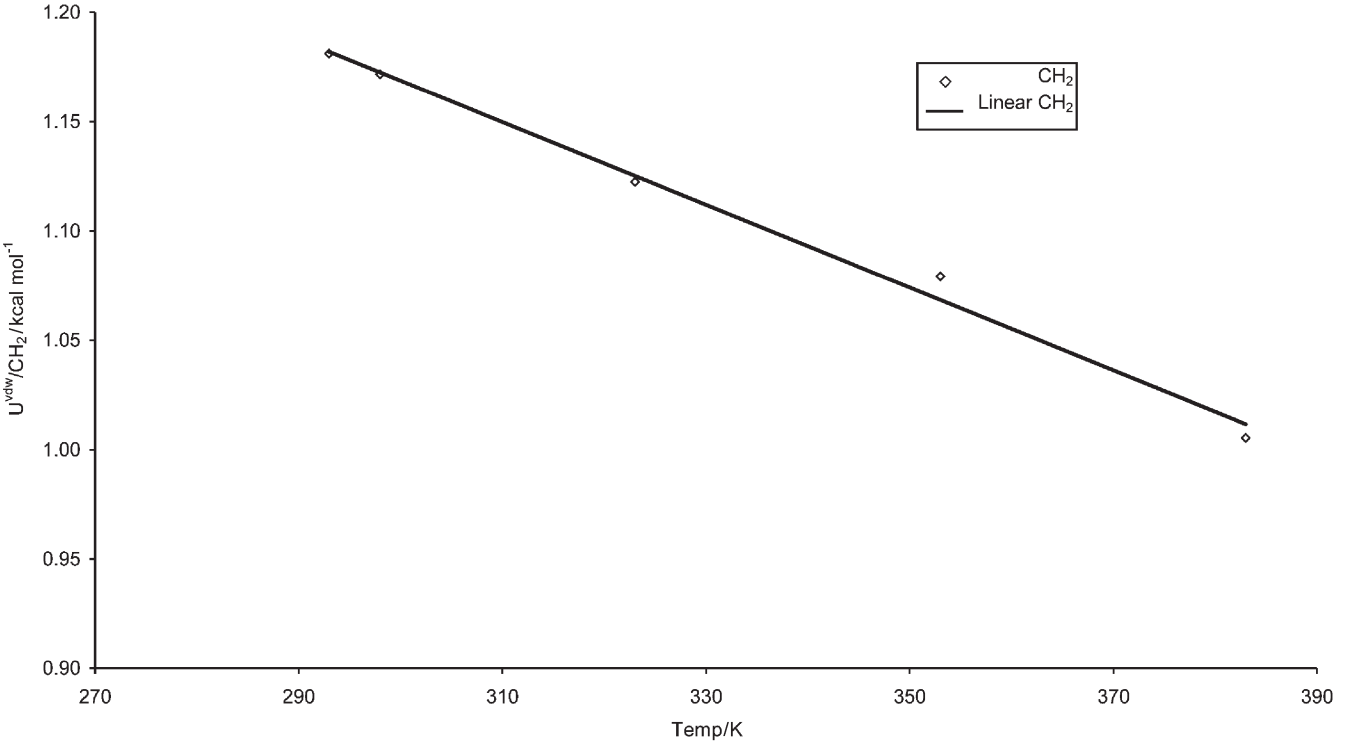

Figure 5. Variation in van der Waals energy of CH_2 with temperature.

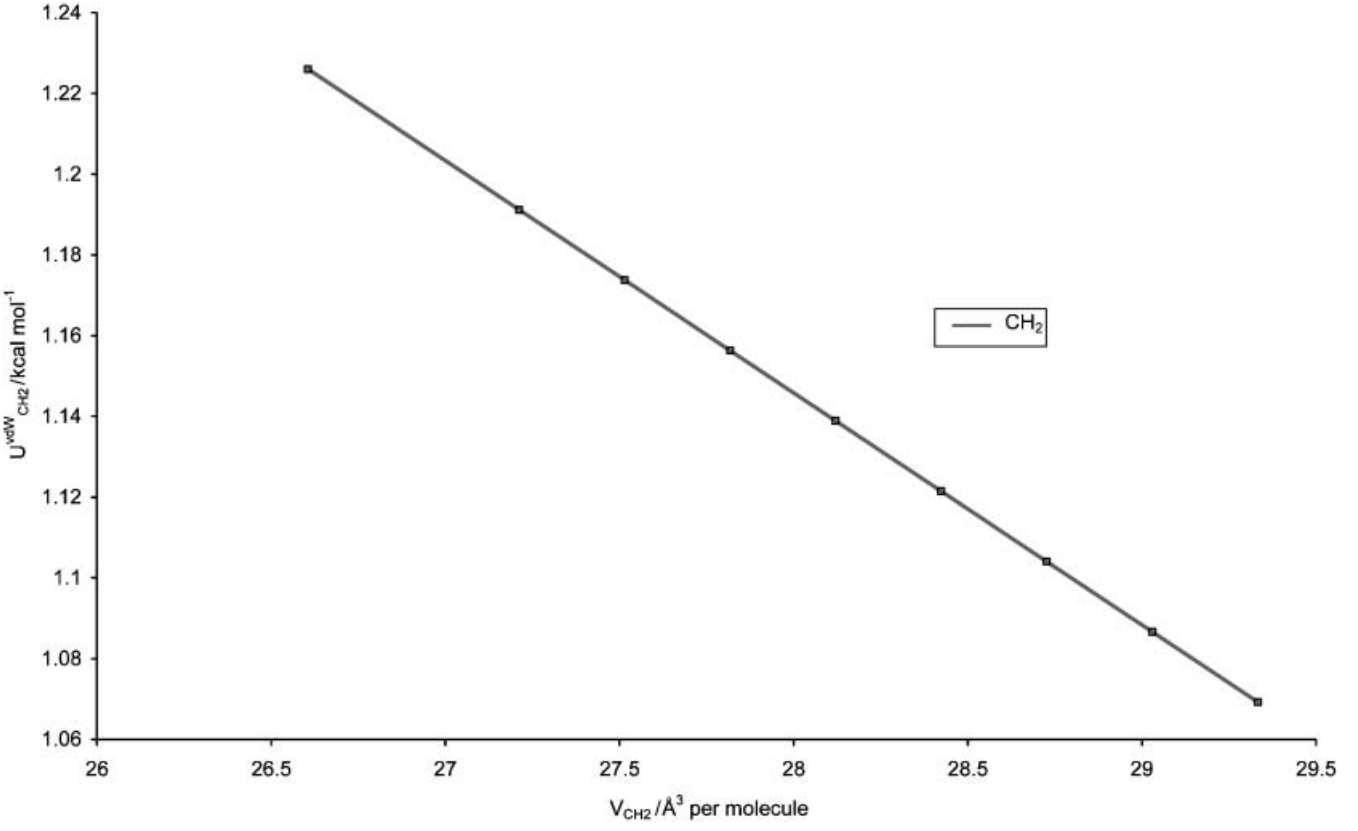

Figure 6. Comparison of respective U^{vdW} and volume change per CH_2 group.

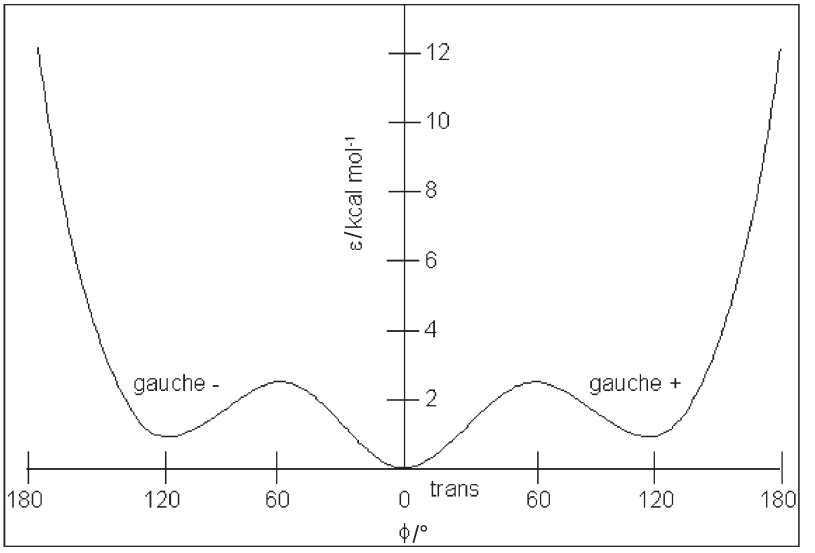

Figure 7. The conformational energy of *n*-butane as a function of the rotation angle ϕ about the central C–C bond.

Assuming the first case, in which all-*trans* exists solely in the crystalline state, we can use equations (14) and (15) to estimate the rotameric energy [6]. In the lamellar phase (i.e. above the gel transition temperature) the average fraction of dihedral bonds in either the g^+ or the g^- states P_g^{liq} is given by the Boltzmann distribution:

$$P_g^{\text{liq}} = \frac{2\exp(\varepsilon/kT)}{1+2\exp(\varepsilon/kT)} \tag{14}$$

where T is absolute temperature, and k is the Boltzmann constant. In the gel state, we assume that all the dihedral bonds exist in the *trans* state, and so none of the *gauche* states are occupied:

$$P_g^{\text{solid}} = 0. \tag{15}$$

The total energy change $\Delta U_{t/g}^{\text{Rot}}$ in the system associated with the shift in the population of the rotational states due to the gel transition is then given by

$$\Delta U_{t/g}^{\text{Rot}} = \varepsilon\left(P_g^{\text{solid}} - P_g^{\text{liquid}}\right) n_{\text{dihedral}} \tag{16}$$

where n_{dihedral} is the number of dihedral angles, ε is the energy difference between the *trans* and *gauche* configurations; and P_g^{liq}, P_g^{solid} are the probability of a *gauche* rotamer existing in the liquid or solid states respectively.

From the work of Flory [14] and Nagle and Wilkinson [6], ε can be shown to be approximately $0.5 \pm 0.1\,\text{kcal mol}^{-1}$. In applying this formula to lecithins, we consider that there are *n*-3 C–C bonds per chain (CH_2–CO_2 and CH_2–CH_3 moieties do not contribute). The values calculated for $\Delta U_{t/g}^{\text{Rot}}$ are given in table 1 below.

4. Results

Figure 8 shows DPPC data for a sequence of five heat/cool cycles (H1, C1, H2, C2, H3, C3, H4, C4, H5, C5). The sample was then held at 50°C for 24 h followed by three cool/heat cycles (reC1, reH1, reC2, reH2, reC3, reH3). Both the main and pretransitions can be clearly distinguished, with the pretransition (from $L_{\beta'}$ to $P_{\beta'}$) occurring over *c*. 31–34°C and the main transition ($P_{\beta'}$ to L_α) at *c*. 41.5°C. Note that the data for the lamellar phase lie very close to the line for the alkanes. It is immediately apparent from figure 8 that successive runs give slightly different data, particularly below the main transition where there is a gradual decrease of molecular volume. This change is much larger than the experimental error. On repeat heat/cool cycles for dodecane the measured volumes were identical within $0.0005\,\text{Å}^3$ per CH_2 group. The curves obtained after 24 h storage of the sample at 50°C show much smaller changes, and may be close to an 'equilibrium' state. We did not record data over longer periods because we feared that slow hydrolysis of the lecithins could occur. Note that we were unable to detect any sign of hydrolysis by thin layer chromatography of the samples after measurement.

Very similar changes are observed for repeated samples. Figure 9 shows the same set of repeated scans for four different samples. Whilst the spread of data for the lamellar phase may indicate some small systematic error in the sample preparation, there is a much larger spread for the gel phases. The general pattern and spread of CH_2 group volumes is always reproducible, and the families of curves are mostly overlapping. Individual curves do not superimpose.

Table 1. Phase transition results for symmetrical lecithins C16:0/C16:0 and C14:0/C14:0.

	C16:0/C16:0		C14:0/C14:0	
Property ($\pm$error, units)	Heating	Cooling	Heating	Cooling
$\frac{dV}{dT}$ (± 0.002 Å^3 °C^{-1}) post T_M	1.114	1.109	0.969	0.959
$\frac{dV}{dT}$ (± 0.002 Å^3 °C^{-1}) $T_P<T<T_M$	1.919	1.894	1.681	1.561
$\frac{dV}{dT}$ pre T_P (± 0.002 Å^3 °C^{-1})	0.989	0.984	0.979	1.137
T_M (± 0.2°C)	42.0	41.5	24.0	23.5
Volume change (± 0.1 Å^3)	53.5[a]	53.0	42.1	41.4
	(57.3)[b]	(56.5)	(42.8)	(42.3)
ΔH main (± 0.01 kcal mol^{-1})	9.10[12]	—	6.21[17]	—
	8.60[16]			
ΔU^{vdW} main (± 0.01 kcal mol^{-1})	3.24	3.21	2.46	2.42
	(3.38)	(3.33)	(2.49)	
ΔU^{Rot} main (± 0.01 kcal mol^{-1})	6.16	6.15	5.08	5.07
ΔU_{Total} (± 0.01 kcal mol^{-1})	9.40	9.36	7.54	7.49
T_P (± 0.2°C)	34.5	30.0	13.5	11.5
Volume change (± 0.1 Å^3)	7.8	7.1	8.7	5.6
	(8.8)	(7.3)	(9.1)	(5.7)
ΔH pre (± 0.01 kcal mol^{-1})	0.45[18]	—	0.30[19]	—
ΔU^{vdW} pre (± 0.01 kcal mol^{-1})	0.46	0.42	0.40	0.33
	(0.52)	(0.43)	(0.53)	(0.33)
ΔU^{Rot} pre (± 0.01 kcal mol^{-1})	—	—	—	—
ΔU_{Total} (± 0.01 kcal mol^{-1})	0.46	0.42	0.40	0.33

[a]Average volume change over all heat/cool cycles.
[b]Parentheses identify maximum measured volume change of all heat/cool cycles.

In order to see the changes more clearly, expanded sections of the curves from figure 8 are given in figure 10. As many others have reported, the width of the main transition (<0.5°C) is far smaller than that of the pretransition (2°C). Moreover, the main transition shows no change in the transition temperature between heating and cooling cycles, whilst the pretransition occurs at *c*. 3°C higher temperature on heating than on cooling. Finally, comparison of the lamellar phase data with those for the alkanes shows that there is a deviation from the alkane behaviour about 2–3°C above the transition temperature for both heating and cooling cycles (note the marked deviation from the alkane data in figure 10 (*a*) at 41–42°C).

Figure 11 compares the volume changes at three different temperatures (5, 37.0, 46.5°C) and across the transitions. The values appear to level out at 5°C following the 50°C storage but there appears to be a very small decrease in volume continuing for the L_α phase (note also the consistent difference between values on heating and cooling cycles). This is not seen in the data for dodecane. For the $P_{\beta'}$ phase it is clear that the volume continues to decrease, even after the third re-heat cycle. The volume change across the main transition appears still to be increasing, mostly due to continuing changes in the $P_{\beta'}$ phase. It is possible that changes in the volume across the pretransition have stabilized after the third re-heat, but there is a significant difference between heating and cooling cycles.

Figure 12 shows a similar data set for DMPC. As before, the sample was equilibrated at 5°C before the first heat/cool cycle, followed by storage at 50°C for 24 h prior to the final three re-cool/heat cycles. Before discussing the data in detail we need to comment on the difference between the CH_2 group volumes in the lamellar phase and those of liquid alkanes. Whilst the slopes of the lines in figure 12 (*a*) are the same at higher temperatures, the data are off-set from each other by *c*. 0.1 Å^3. This appears to be a real difference that occurs for short chain saturated lecithins (C_n, $n<16$). We observe an even larger deviation for the $C_{12}C_{16}$ compound, while $C_{14}C_{16}$ and $C_{16}C_{14}$, are very similar to DPPC [15]. The effect is only *c*. 3 Å^3 in a total volume of *c*. 1110 Å^3 per molecule. As yet the origin of this difference is not resolved. One possibility is that there is a change in the head group volume for short chain derivatives. Short chain lecithins (C_n, $n<10$) form a micellar solution at low concentrations in water rather than a lamellar dispersion. Clearly, some part of the detailed arrangements of the head groups at the water interface must change as the chain length decreases because the surface is curved rather than flat. This could be accompanied by conformational changes giving the observed volume change.

The pretransition and main transition are clearly visible, as are the differences in transition temperatures

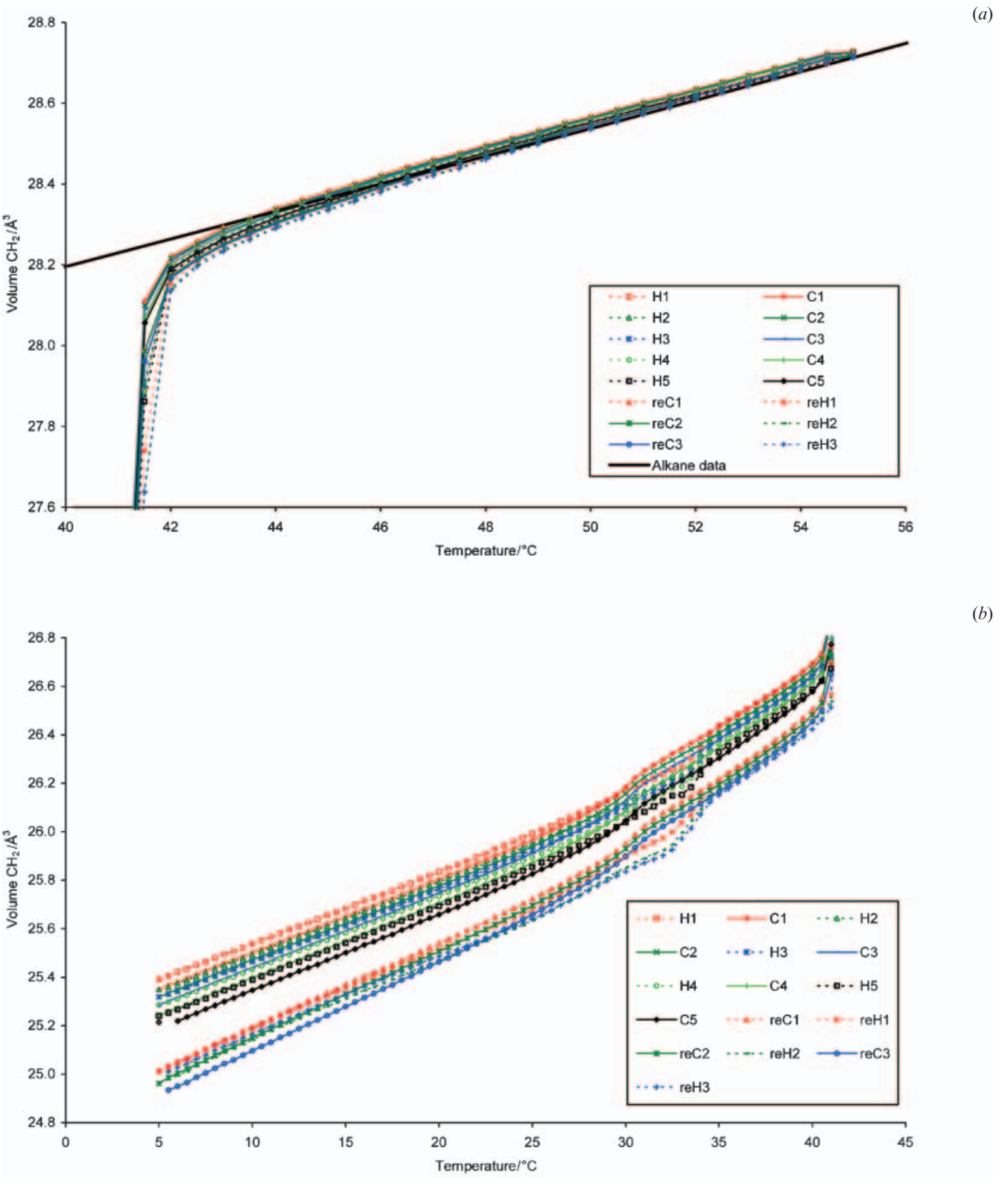

Figure 8. (*a*) CH_2 group volume as a function of temperature above T_M; (*b*) CH_2 group volume as a function of temperature below T_M. The curves show measurements for a sequence of five heat/cool cycles (H1, C1, H2, C2, H3, C3, H4, C4, H5, C5) after initial equilibration at 5°C for 24 h. The sample was then held at 50°C for 24 h followed by three cool/heat cycles (reC1, reH1, reC2, reH2, reC3, reH3).

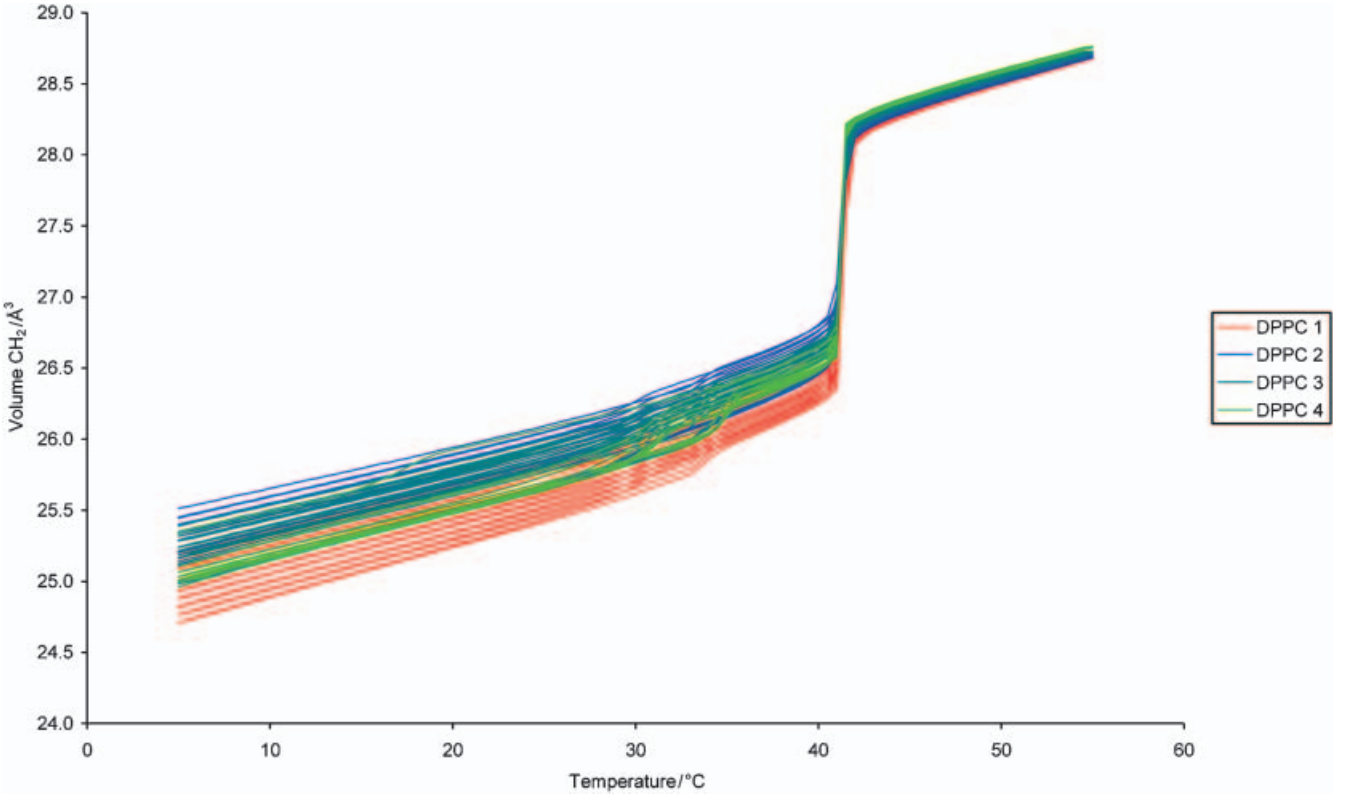

Figure 9. Repeated heating and cooling curves for four different DPPC samples.

between the heating and cooling curves for the pretransition. Also, there is a much larger deviation of the molecular volume from the linear behaviour, just above the main transition for DMPC, than we observe for DPPC. This occurs up to about 10°C above the transition. With the exception of the first heating cycle, the changes in volume between different temperature cycles are far smaller but in the same direction as for DPPC; they are of a similar magnitude in all three phases. This large difference between the first and subsequent cycles is reproducible on repeated samples, but does not reappear if the sample is kept at 5°C for 24 h after the other measurements have been made. Note also that the first heating curve has the pretransition temperature *c*. 2.5°C higher than for subsequent heating cycles. It is quite clear that the initial relaxation of the DMPC system is much faster than that for DPPC. The changes in volume across phase transitions, transition temperatures, enthalpies and other data are summarized in figure 13 and table 1.

5. Discussion

Comparing the two data sets, it is clear that the molecular volumes of the two compounds in the different phases (lamellar, gel and second gel) are very similar. We conclude that the lamellar phase is a very different state from the two lower temperature phases, but that neither of these is close to being considered as a crystalline solid, where the CH_2 group volume is expected to be *c*. 24 $Å^3$. Thus both are gel (L_β) phases. In fact our results indicate that current models of gel phase structures, which consider all the alkyl chains to pack into a very regular long range structure, are misleading. Whilst there can be no doubt that the hydrocarbon chain configuration is mainly in the all-*trans* configuration, the dependence of molecular volume on sample history suggests that the detailed packing of the chains does not adopt one unique structure. Rather we should consider there to be a varying fraction of *gauche* configurations. We discuss this further below.

Table 1 summarizes a range of data for DPPC and DMPC, from this study and from the literature. We have included our calculations of the various contributions to the transition enthalpies, the measured enthalpies from elsewhere [12, 16–19], and our measured coefficients for the changes in volume with temperature (dV/dt). For the pretransition, the values of ΔH estimated from the measured ΔV are in good agreement with literature values [18, 19]. Note that we expect different ΔH values on heating from those on cooling

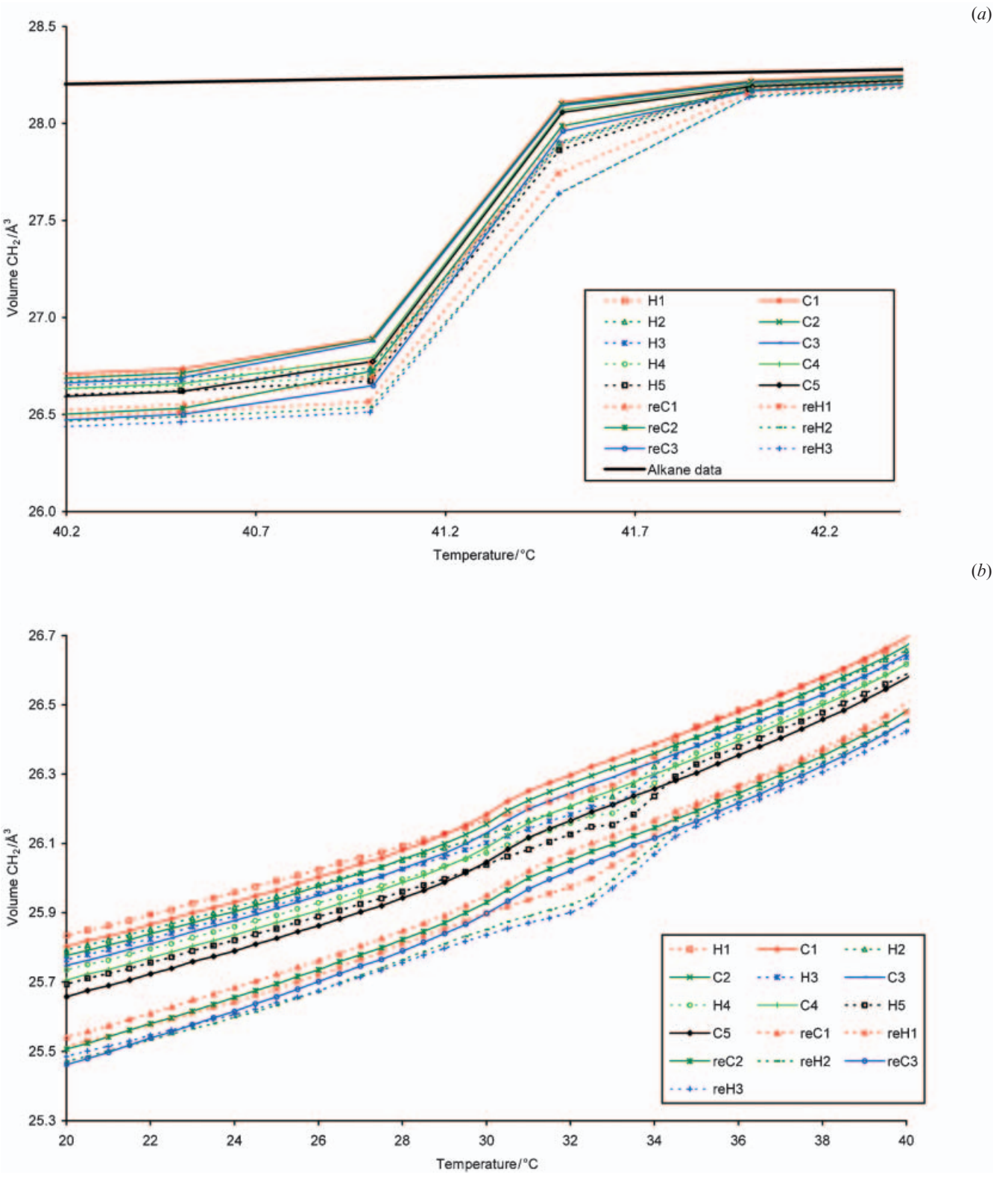

Figure 10. Expanded data sets for (*a*) main and (*b*) pretransitions of DPPC. H1, C1, etc. as for figure 8.

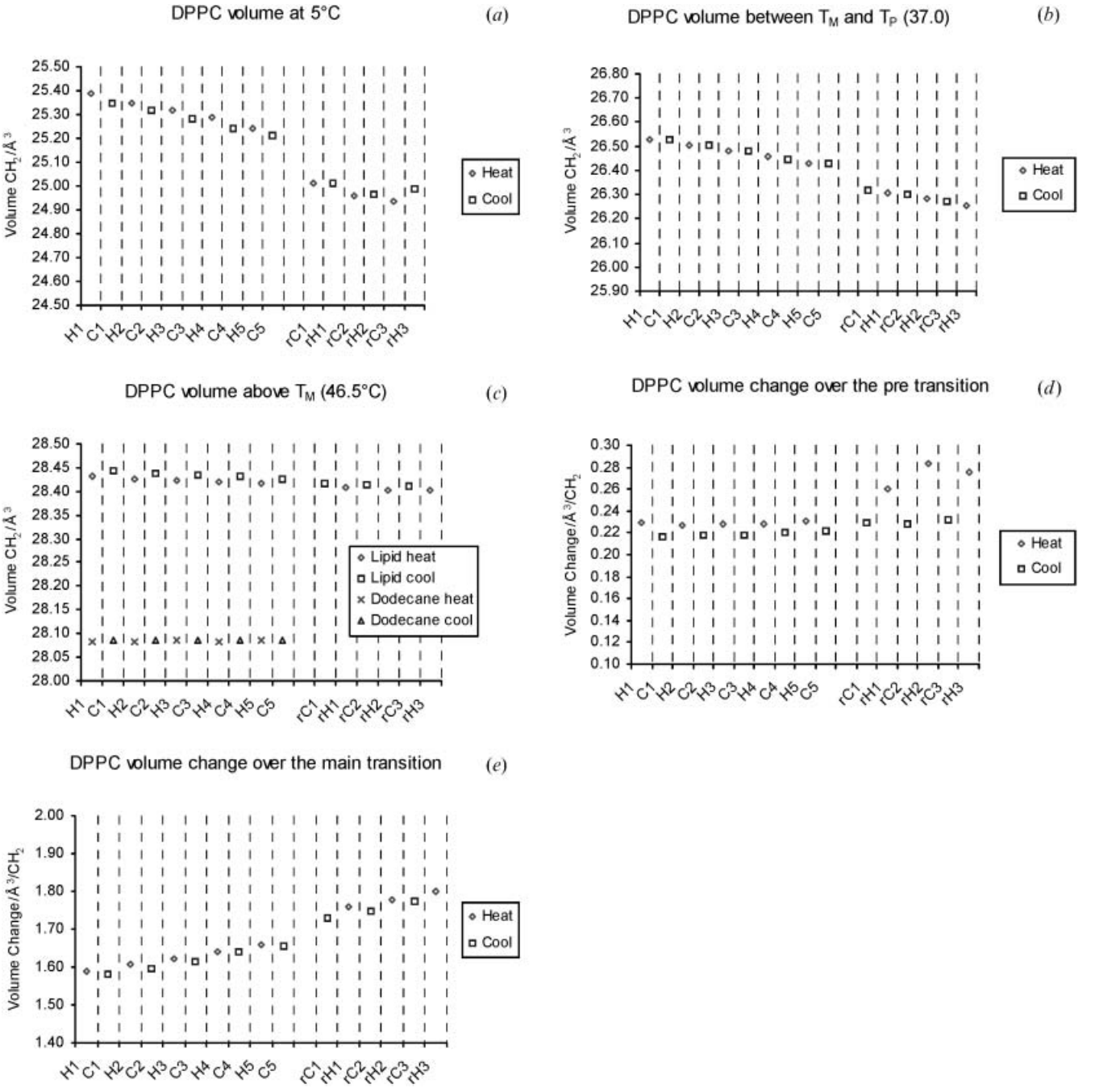

Figure 11. (*a*) Volume per CH_2 group for DPPC in repeated cycles at 5°C. (*b*) Volume per CH_2 group for DPPC midway between T_M and T_P. (*c*) Volume per CH_2 group for DPPC in the L_α phase at 46.5°C. (*d*) Volume per CH_2 group for DPPC across T_P. (*e*) Volume per CH_2 group for DPPC across T_M. H1, C1, etc. as for figure 8.

because we consistently measure different ΔV values. The results from cooling curves are not given in the literature. In considering the main transition, the contributions ΔU^{vdW} and ΔU^{Rot} are evidently both significant contributors to the enthalpy. However, while the calculated ΔH for DPPC is close to the observed value, that for DMPC is an overestimation. It is likely that the assumed 'all-*trans*' configuration for chains just below the main transition is responsible for this. It is highly probable that some configurational disorder is still present below the main transition. We have tabulated the values of (dV/dt) for the three different phases. It is clear that the value for the $P_{\beta'}$ phase is larger than the other two phases, as would be expected

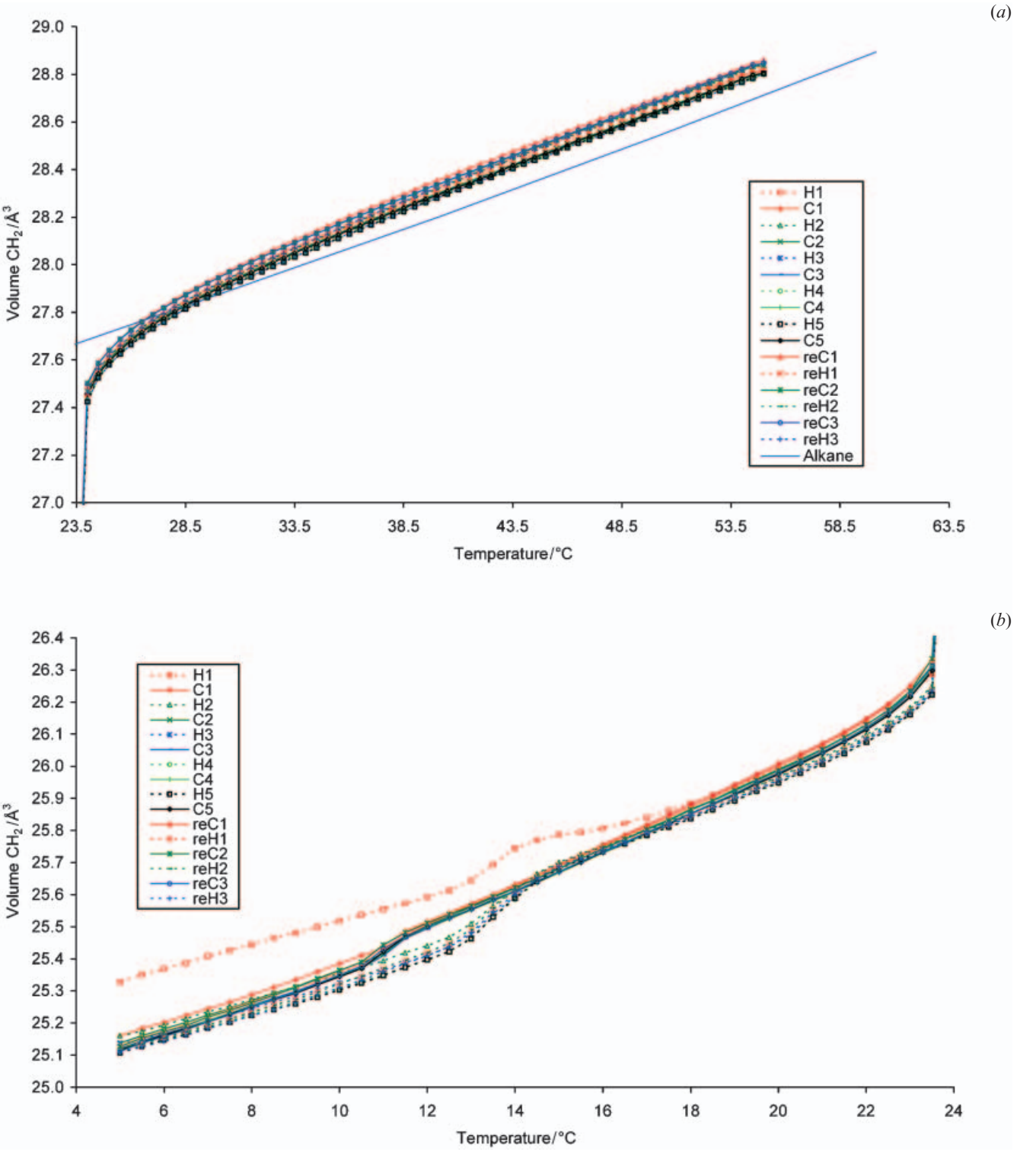

Figure 12. CH_2 group volume for DMPC as a function of temperature: (*a*) above T_M; (*b*) below T_M. The curves show measurements for a sequence of five heat/cool cycles (H1, C1, H2, C2, H3, C3, H4, C4, H5, C5) after initial equilibration at 5°C for 24 h. The sample was then held at 50°C for 24 h followed by three cool/heat cycles (reC1, reH1, reC2, reH2, reC3, reH3).

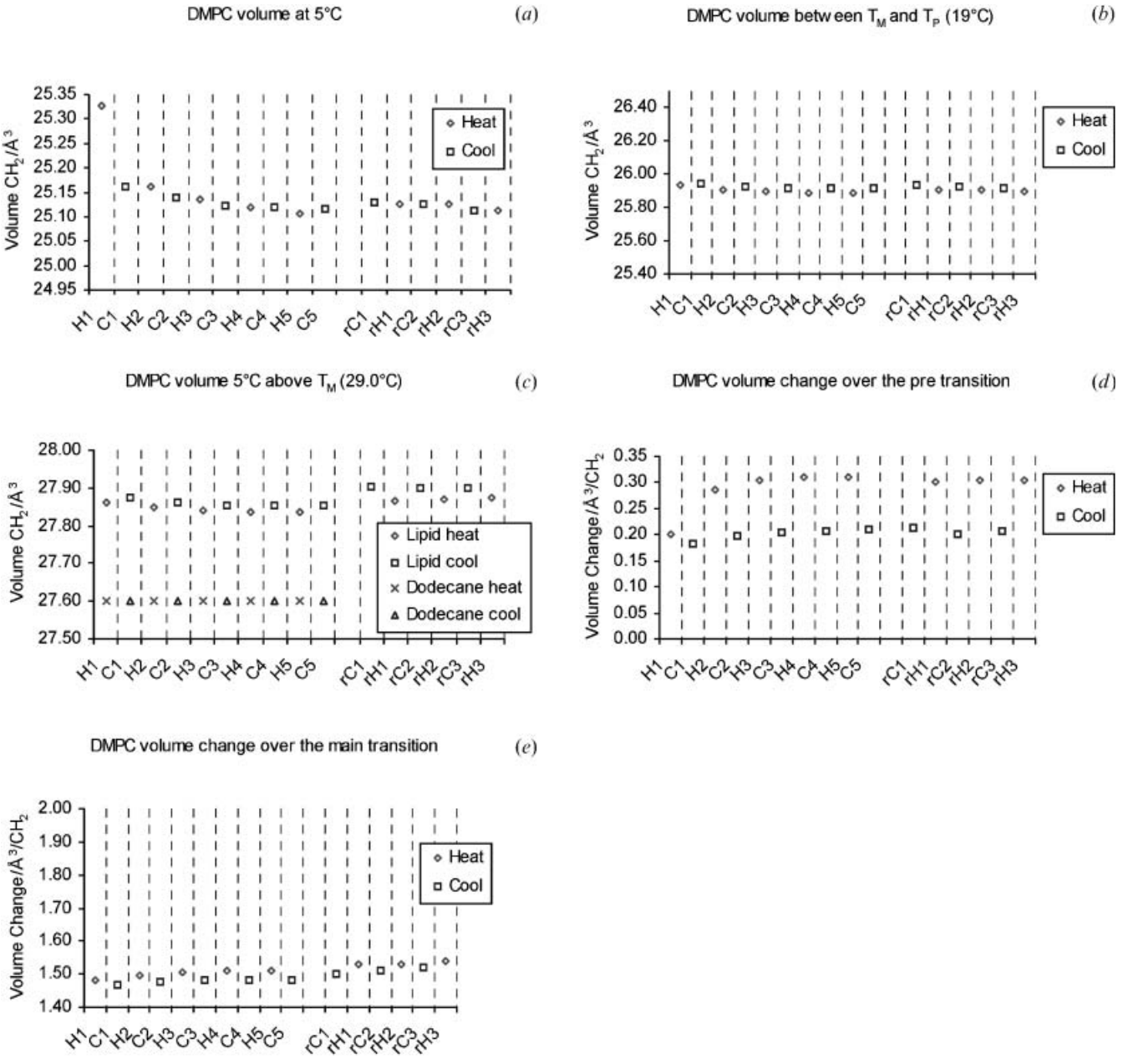

Figure 13. (*a*) Volume per CH_2 group for DMPC in repeated cycles at 5°C. (*b*) Volume per CH_2 group for DPPC midway between T_M and T_P. (*c*) Volume per CH_2 group for DMPC in the L_α phase at 29.0°C. (*d*) Volume per CH_2 group for DPPC across T_P. (*e*) Volume per CH_2 group for DPPC across T_M.

if there were a larger increase in the alkyl chain disorder within this phase. There should also be an increased heat capacity for this phase; differential scanning calorimetry measurements are underway to investigate this possibility.

The phase sequences experienced by DMPC and DPPC are the same $\left(L_{\beta'} \Leftrightarrow P_{\beta'} \Leftrightarrow L_\alpha\right)$, but with transition temperatures and volume changes that depend on sample history. We suggest that instead of being regarded as similar to crystalline phases having a well defined and rigid molecular configuration, the gel state be regarded as having more of the character of a liquid phase (except that there is essentially no lateral diffusion), where a significant minor proportion of the molecules have some configurational freedom. Thus, a range of possible molecular configurations can occur, which has a consequence for the nature of the phase transitions. Ostwald's rule on polymorphism states: *A crystallizing system progresses from the supersaturated state to equilibrium in stages, each stage representing the*

smallest possible change in free energy [20]. Thus, if there are a series of possible gel states, the highest free energy form is the one that occurs on cooling. It is well known that for a series of crystalline polymorphs the melting points are lower, the higher the free energy of the phase. Here we consistently observe that the pretransition occurs at a lower temperature on cooling than on heating, exactly as would be expected if the $L_{\beta'}$ phase formed on cooling were a higher free energy state than the state that exists after cooling to 5°C. Similarly, the main transition occurs 0.5°C lower on the cooling cycle than on the heating cycle. Thus the $P_{\beta'}$ phase when formed on cooling is not in the lowest free energy state. These differences in transition temperatures are observed widely for gel phases of many different surfactants [21]. Hence we suggest that because gel phases all contain a variable range of alkyl chain configurations (mostly all-*trans*), on cooling they will initially form a structure representing the smallest possible change in free energy from the high temperature phase. Inevitably, this will be followed by a slower relaxation to a more stable state.

It is noteworthy that the chain configurations of the lamellar phase equilibrium state are likely to be very different from those of the two gel phases. We consider that the main relaxation process observed here is relaxation within the lamellar phase. It might be that this moves the gel states further from their real equilibrium configuration. The first heating run for DMPC gives a higher pretransition temperature than those of subsequent runs, indicating that the initial state is the closest to equilibrium. This is despite the density gradually reducing through the temperature cycles. The system does not approach the initial state again — remaining far from the second gel state equilibrium.

One of the most surprising results of this work is the observation in the lamellar phase of the pre-T_M volume reduction well above the transition temperature — up to 10°C for DMPC. It is not surprising that the effect is larger for DMPC than for DPPC because generally these phenomena are larger, the weaker the transition (i.e. the smaller the ΔH). But the magnitude of the effect (*c.* 0.2 Å^3 per CH_2 group) is approximately 10% of the change measured at the transition. This implies that there is a significant fraction (10%) of lipid molecules in an all-*trans* configuration just above the transition, independently of whether the temperature is approached on heating or cooling. Because the phase transition itself is a collective property involving many molecules, it seems likely that this pretransitional volume reduction also involves groups of molecules. Thus we appear to have evidence for the spontaneous formation of 'patches' where the molecules are in an all-*trans* configuration. Clearly, this is worthy of further investigation — it appears to resemble the occurrence of lipid patches in membranes [2–4]. Whilst there are numerous differences from real membranes, including the absence of mixed lipid chains, the occurrence of ordered patches in pure lipids having mixed chains, and in mixed lipids, is certainly worth exploration as a contribution towards the understanding of membrane behaviour.

Finally we consider the polymorphism of the lipids and the molecular mechanism of the relaxation phenomena. It is clear that the state of DMPC at the beginning of the measurements is certainly a non-equilibrium one for the lamellar phase, despite initial dispersal in water, and being stirred for 24 h at 55°C. Each cycle of density measurements takes about 14 h. Thus the complete measurement set takes 5 days, after which it is not possible to reproduce the original curve by storage at 5°C for 24 h. We believe that the most likely explanation involves very slow equilibration of the different alkyl chain configurations for the head group/glycerol moieties. The initial sample is prepared from a dry powder. On contact with water the powder rapidly (seconds) absorbs water to give a gel or lamellar phase. At no time does the majority of the material exist as monomers. Thus the molecular configuration will be the one most easy to access from the solid state, not the configuration that has the lowest free energy in the lamellar phase. It would appear that for DMPC this initial state is further from the lamellar equilibrium state than the initial state of DPPC.

6. Conclusions

Density measurements were performed on dispersions of DPPC and DMPC in water in a series of successive heating and cooling experiments. These measurements revealed the presence of slow relaxation processes within the gel phases of lecithins in water, probably as a result of slow equilibration of alkyl chain configurations. Similar relaxation processes occur in lamellar phases, but are much faster and weaker in magnitude. We expect that all gel phases formed by cooling a lamellar phase (not just those formed by lecithins) are likely to exhibit slow relaxation phenomena, particularly from longer chain, di-alkyl surfactants.

Well above the main transition in the lamellar phase, the volumetric behaviour of the lipid tail volume is essentially the same as that of a hydrocarbon liquid. However, just above the main transition temperature, it differs substantially from the hydrocarbon liquid, suggesting that a significant concentration of lipid in

the lamellar phase exists in the 'gel-state' just above the main transition. This may be related to the formation of lipid patches in biological membranes.

Finally, from the decomposition of the enthalpies of the 'pretransition' and the main transition, we find that the 'pretransition' mainly arises from a change in the van der Waals interactions between the lipid molecules, while the main transition arises from changes in both the van der Waals interactions and the chain rotameric (configurational) energies.

Acknowledgements

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