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Lateral diffusion of small solutes and partition of amphipaths in defect structures of lipid bilayers

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The excimer formation technique has been applied to investigate the mechanism of the lateral diffusion in the crystalline $P_{\beta'}$ phase of dipalmitoylphosphatidylcholine vesicles. This became possible at low pyrene concentrations. From the shape of phase-transition curves, from the effect of pressure up to 150 bar and from the perturbation induced by cholesterol, we conclude that the free volume model could also be applied to the $P_{\beta'}$ phase. However, the diffusion is thought to occur in defect structures that are considered to form fluid pathways between domains of crystalline lipid. Partition coefficients of amphipaths provide a basis for testing for the role of defects. The amphipath chlorpromazine partitions into fluid membranes with a partition coefficient, k_p, of 3200, into the crystalline phase with $k_p = 200$ but into the $P_{\beta'}$ phase with a value of $k_p = 800$. This again gives rise to the assumption that the $P_{\beta'}$ phase contains fluid domains that behave like the fluid L_α phase and make up about 10–20% of the total amount of lipid in the bilayer. Cholesterol is known to interfere especially with defect structures in the $P_{\beta'}$ phase. It fills up the gaps, and therefore reduces the partition coefficient to almost zero in the $P_{\beta'}$ phase.

Introduction

The investigation of translational diffusion of lipids and proteins in artificial lipid bilayer membranes is of considerable interest in obtaining models for the transport mechanism in biological membranes. Various experimental techniques together with theoretical considerations have been reviewed recently [1]. The excimer formation technique [2] led to the application of the free volume model [3] to describe the lateral diffusion of small molecules in fluid bilayer membranes [4]. The applicability of this model was further supported by considering the pressure effect on lateral diffusion [5] but also by monolayer experiments [6] or by the use of the 'fluorescence recovery after photobleaching' technique [7]. Very recently, the latter technique was also applied to investigate lateral diffusion of lipids and proteins in the $P_{\beta'}$ phase, which is between the pre- and the main transition temperature of the corresponding lipid [8]. This so-called ripple phase is characterized by defect structures [9] which may be considered as fluid pathways traversing rigidified domains of crystalline lipid. The question arises whether the diffusion process in defect structures may as well be described by the free volume mechanism.

A prerequisite for such a mechanism is the presence of volume exceeding the Van der Waal's volume of the molecule. This is fulfilled in the fluid phase. From diffusion measurements it was concluded that 10–20% of the lipids are present in defect structures in the $P_{\beta'}$ phase, whereas this amount makes up less than 5% in the crystalline L_β phase [8].

Abbreviation: DPPC, dipalmitoylphosphatidylcholine.

Fluid bilayer membranes are able to take up amphipaths from the aqueous phase. The partition coefficient is a measure for the uptake of a given amphipath such as the positively charged drug chlorpromazine. In a recent paper we reported that chlorpromazine solubility depends critically on the physical state of the membrane [10]. Now, we have focussed on the $P_{\beta'}$ phase in the absence and in the presence of cholesterol. The value of the partition coefficient is regarded as a measure for the fraction of fluidized lipid in which diffusion might occur. The effect of cholesterol, which is known to disturb defect structures especially [11], is discussed.

Materials and Methods

Lipids and probes

DPPC was obtained from Fluka (Neu-Ulm, F.R.G.), checked by TLC and used without further purification. Cholesterol and chlorpromazine were from Serva (Heidelberg, F.R.G.). Pyrene (Fluka) was highly purified by repetitive zone melting.

Vesicle preparation

Diffusion measurements were performed with moderately sonified vesicles. Lipids, cholesterol and pyrene were dissolved in chloroform. The solvent was removed at 50°C by a stream of nitrogen and by subsequent evaporation in a vacuum oven for several hours. 1 mM Tris/HCl buffer (pH 7.0) was added and the samples were sonicated for 10 min at 50°C in a bath sonifier (Bandelin, Sonorex RK 255). The final lipid concentration was 1 mg/ml. Large unilamellar vesicles for the determination of the partition coefficients were prepared by a modification of the method of Szoka and Papahadjopoulos [12]. A 25 mM solution of phosphatidylcholine or phosphatidylcholine/cholesterol in chloroform/buffer (2 : 1, v/v) was sonicated briefly (Branson sonifier) until the mixture became a homogeneous dispersion. In a typical experiment, 17 mg lipid was dissolved in 1 ml chloroform/buffer. The buffer solution throughout the experiments was 140 mM NaCl/10 mM Tris-HCl (pH 7.0). The organic solvent was then removed at 50°C under reduced pressure by a rotary evaporator. The final aqueous suspension was washed three times by centrifugation (12000 $\times g$, 15 min) in 20 vol. buffer. The final pellet was resuspended in 10–30 ml buffer solution to make up a lipid concentration of 0.2–1.0 mg/ml. Typical vesicle size was between 1000 and 2000 Å.

Spectroscopic methods

Fluorescence measurements were performed with a fluorescence spectrometer equipped with two sets of monochromator/photomultiplier systems arranged perpendicular to the irradiation beam. Pyrene was excited at 335 nm, the monomer emission (I) and the excimer emission (I') were measured simultaneously at 394 and 470 nm, respectively. The intensity ratio, I'/I, was calculated concurrently with temperature by a microcomputer. Samples were kept in 1 cm quartz cuvettes in a specially constructed thermostatically controlled metal block equipped with pressure-resistant quartz windows. Temperature was measured by a thermocouple in the sample. Pressure was attained from a helium source pressurized to 200 bar. Residual air was removed from the system by pressurizing and venting it several times. Absorbance measurements were carried out with a Varian Cary 118 spectrometer.

Determination of partition coefficients

1 ml of vesicle suspension was incubated with chlorpromazine at $2.8 \cdot 10^{-5}$ M. Vesicles were incubated at the desired temperature for 30 min and centrifuged for 15 min at 12500 $\times g$. After centrifugation, the remaining concentration of chlorpromazine in the supernatant was determined from the absorbance of chlorpromazine at 254 nm (E = 31000 $cm^{-1} \cdot M^{-1}$). The partition coefficient, k_p, was calculated from:

$$k_p = \frac{C_{CPZ(m)}}{C_{CPZ(b)}} \cdot \frac{V_b}{V_m}$$

where C_{CPZ} is the concentration of chlorpromazine in membrane (m) or in buffer (b) and V is the volume of membranes (m) and of the buffer (b), respectively.

In a typical experiment, a 1 ml suspension of membranes contained $2.8 \cdot 10^{-5}$ M chlorpromazine. The concentration of chlorpromazine in the membranes was calculated from total chlor-

promazine concentration minus chlorpromazine concentration in the buffer solution.

Membrane volume, V_m, was taken to be the lipid compartment of the membrane. It was calculated from phosphate analysis and known cholesterol content and a density of 1 g/ml for lipids. The partition coefficient was corrected for lipid remaining in the supernatant.

Lipid concentration was calculated from phosphate analysis. First, vesicle suspensions were heated at 200°C for 1 h with a few drops of 70% perchloric acid. Phosphate determination was performed by a modification of the method of Chen et al. [13]. After cooling of the samples, 3.5 ml distilled water, 0.45 ml 70% perchloric acid, 0.5 ml 2.5% ammonium molybdate and 0.5 ml 10% ascorbic acid were added. After 5 min at 100°C the absorbance was measured at 800 nm.

Results

Effect of pressure on phase-transition curves obtained by the excimer technique

Phase-transition curves obtained by plotting the excimer-to-monomer fluorescence intensity ratio I'/I versus temperature are given in Fig. 1 at atmospheric pressure (a) and at 50 (b), 100 (c) and at 150 (d) bar. Pyrene concentrations were reduced from 20 mmol/mol lipid (A) via 5 (B) and 2.5 (C) to 1.25 (D) mmol/mol lipid. In the absence of external pressure (phase-transition curves a), we clearly observe a decrease in I'/I at the pretransition temperature, which is $t_p = 33$°C in DPPC membranes [14]. A further decrease is clearly observable at the main transition temperature, $t_t = 41$°C. In the fluid phase (above the phase-transition temperature) I'/I increases with

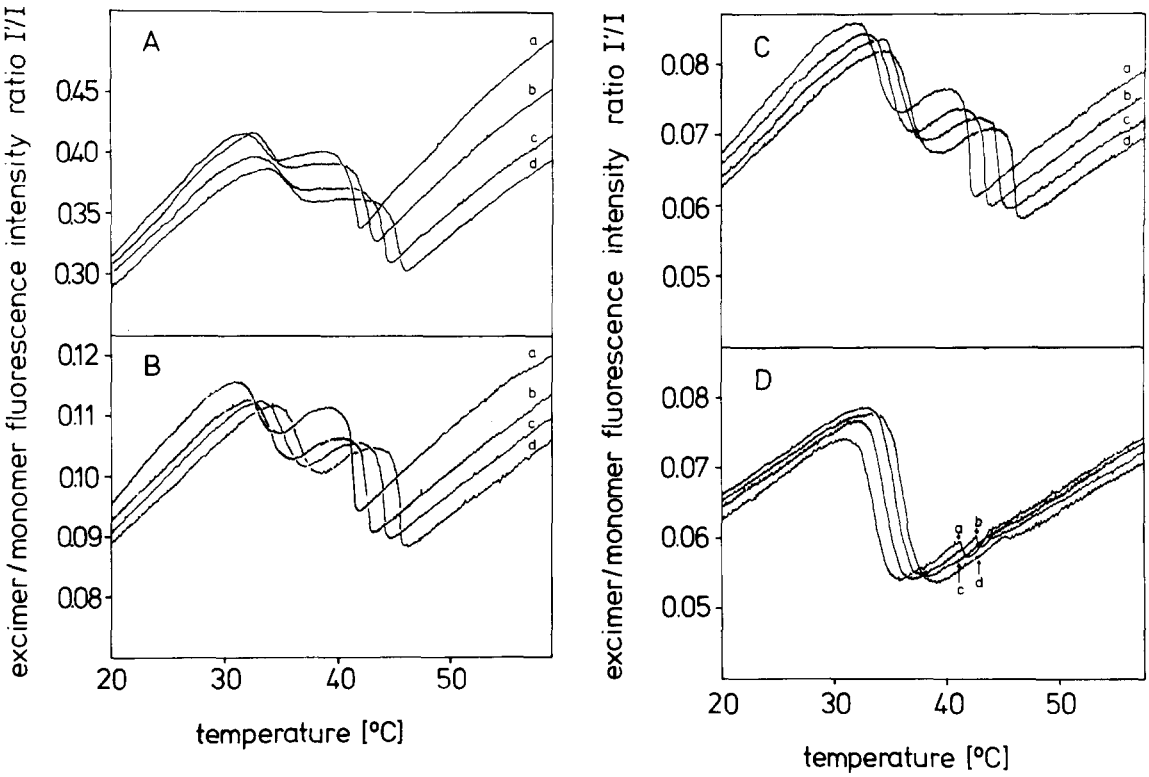

Fig. 1. Thermotropic phase-transition curves of dipalmitoylphosphatidylcholine vesicles obtained by the excimer formation technique. Curves were taken automatically by following the change in the excimer-to-monomer fluorescence intensity ratio with increasing temperature at atmospheric pressure (curves a), at 50 (curves b), at 100 (curves c), and at 150 (curves d) bars. The pyrene concentration was: 20 (A), 5 (B), 2.5 (C) and 1.25 (D) mmol/mol lipid.

increasing temperature due to an increasing collision probability as a consequence of the decreasing viscosity in the hydrophobic core of the membrane [15]. This is the temperature range within which diffusion coefficients can be determined by the excimer technique.

With decreasing pyrene concentration (Fig. 1A–D) the pretransition step becomes more pronounced and is solely visible at 1.25 mmol/mol lipid. It should be pointed out that at this low probe concentration the broad excimer band is indistinguishable from long wavelength branch of the monomer band which slightly covers the range around 470 nm.

Thus the change in a calculated I'/I ratio at very low pyrene concentrations is due mainly to a change in the monomer intensity. This is documented in Fig. 2. The I'/I ratio decreases linearly with decreasing pyrene concentration down to 5 mmol/mol lipid and at atmospheric pressure extrapolates to zero, as is expected for a diffusion-controlled process. At even lower concentrations the I'/I value remains unchanged. Nevertheless, the change in the monomer intensity is measurable and may be related to the intensity at 470 nm, which is the maximum of the excimer emission at $c > 5$ mmol/mol lipid.

Another interesting feature of the phase-transition curves taken at low pyrene concentration is the increase in I'/I with temperature between 33 and 37°C which is the temperature range of the $P_{\beta'}$ phase (e.g., Fig. 1B and C). This increase in I'/I with temperature is well comparable to the slope of the I'/I dependence at $t > t_t$ and at $t < t_p$. It is less pronounced in vesicles containing higher amounts of pyrene (Fig. 1A).

Moreover, we calculated the increase of the pre- (t_p) and the main (t_t) transition temperature with pressure increment. Mid points of the corresponding steps in Fig. 1B and C were taken as transition temperatures. Values obtained are $dP/dt_p = 44 \pm 3$ bar/K for the pretransition and $dP/dt_t = 35 \pm 1$ bar/K for the main transition. Curves shown in Fig. 1A were not used due to considerable broadening of the phase transition by the probe itself, curves shown in Fig. 1D were not used because of the missing main transition.

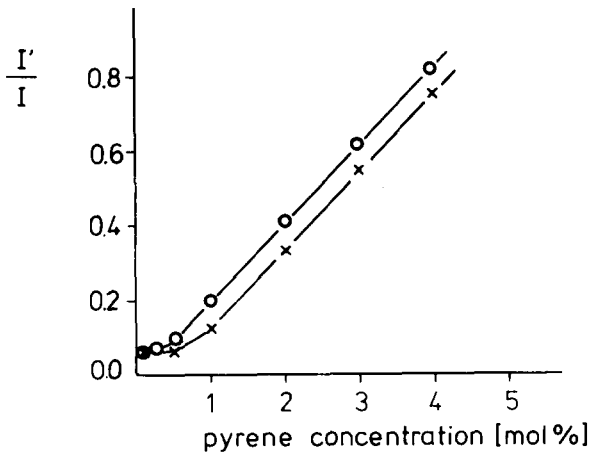

Fig. 2. The excimer/monomer intensity ratio I'/I, of pyrene taken at 50°C in dipalmitoylphosphatidylcholine vesicles is given as function of pyrene concentration. Results at atmospheric pressure (○) and at 150 bars (×) are compared. Note the deviation from the linear extrapolation to zero pyrene concentration at 5 mmol pyrene per mol lipid.

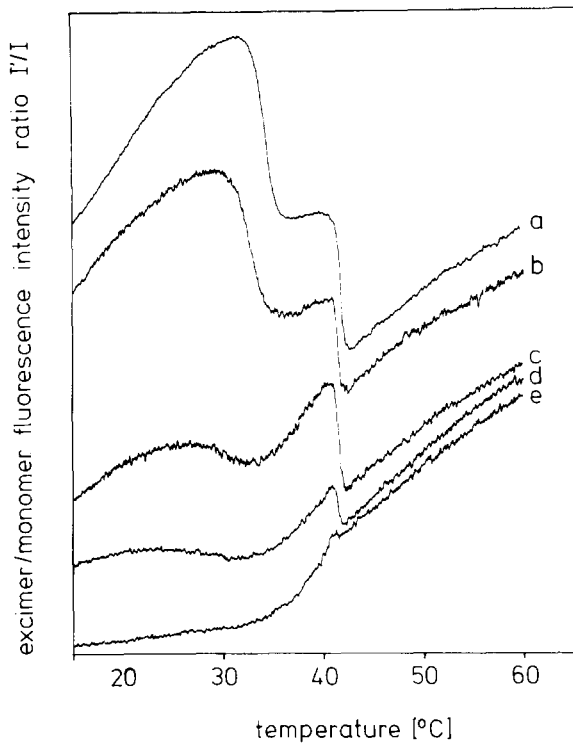

Fig. 3. Thermotropic phase-transition curves of dipalmitoylphosphatidylcholine vesicles using 10 mmol pyrene/mol lipid with increasing amounts of cholesterol: (a) cholesterol-free, (b) 10, (c) 30, (d) 50, (e) 100 mmol cholesterol per mol lipid. The curves are shifted along the ordinate for clarity of presentation.

The effect of cholesterol

Phase-transition curves of DPPC bilayer vesicles containing different amounts of cholesterol were taken at low pyrene concentration (Fig. 3). Up to a cholesterol/lipid ratio of 0.03 mol/mol we observe a drastic decrease of the pretransition step. With increasing amount of cholesterol the main transition determined by the decrease in I'/I around $t = 40°C$ also disappears and changes to a broad increase in I'/I between 33 and 40°C in DPPC membranes containing 0.1 mol cholesterol/mol lipid.

Partition coefficients of chlorpromazine

The solubility of chlorpromazine represented by the partition coefficient was determined with temperature in pure DPPC and in DPPC membranes containing cholesterol (Fig. 4). Here we focus on the $P_{\beta'}$-phase. The partition coefficient, k_p, increases from 200 to 800 at the pretransition temperature. A further increase in k_p to about 3000 was observed upon increasing the temperature above the main transition temperature. Cholesterol reduces the k_p values of chlorpromazine. At a cholesterol content of 0.03 mol/mol, k_p is not significantly changed at $t > t_t$ and $t < t_p$. However, in the temperature range between t_p and t_t k_p decreased from 750 to 400 at 32°C. The pretransition step diminishes. At high amounts of cholesterol (0.2 mol/mol), k_p decreases to almost zero at low and to about 1000 at high temperatures, as was discussed earlier [10].

Discussion

The excimer forming technique has so far been used mainly to determine lateral diffusion coefficients in a fluid lipid phase at probe concentrations between 10 and 30 mmol/mol lipid. Here we used lower pyrene concentrations in order to obtain information about the $P_{\beta'}$ phase. This concentration range cannot be used to calculate diffusion coefficients. Fig. 2 gives the concentration dependence of the excimer/monomer fluorescence intensity ratio. The experimental results deviate from the linear extrapolation to zero pyrene concentration at 5 mmol/mol lipid, which is easy to understand by a simple consideration. Assuming a lateral diffusion coefficient of $D \approx 10^{-7}$ cm^2/s and a lifetime of the excited monomer of $\tau \approx 200$ ns [16], we may estimate the average square displacement from $\bar{x}^2 = 4\ Dt$ in a two-dimensional lattice. This yields $\bar{x}^2 \approx 800$ Å^2. The average lipid area occupied by one pyrene molecule at a molar ratio of 10 mmol/mol lipid is 600 Å^2. Therefore, the probability of forming an excimer is finite at this or even higher probe concentrations (e.g., Fig. 1A, 20 mmol/mol lipid), but negligible at lower concentrations.

The aim of the present paper is to illuminate the rôle of defect structures in the $P_{\beta'}$ phase for the uptake and the transport of small solutes or amphipaths. The $P_{\beta'}$ phase, however, may easily be disturbed by the incorporation of solutes [17]. We therefore used very low pyrene concentrations to measure thermotropic phase-transition curves, yielding two results: the pretransition becomes more pronounced at low pyrene concentrations, which finally leads to the complete disappearance of the main transition; the excimer/monomer intensity ratio increases in the temperature range between the pre- and the main transition temperature, with a slope comparable to the temperature dependence of I'/I above the phase-transition temperature.

From earlier photobleaching experiments [8] we know that defect structures exist in lipid bilayers in the L_β and in the $P_{\beta'}$ phase. The relative amount of lipid within these defects was found to be about 5% in the L_β and about 15–20% in the $P_{\beta'}$ phase. These results allow us to interpret the present data. Pyrene is thought to be dissolved mainly within the defects, leading to an increased local concentration below the pretransition temperature. The collision rate within these defects will then increase with increasing temperature. At the pretransition temperature, t_p, the amount of fluidized lipid increases to about 15–20% of the total lipid, thus leading to a decreased local pyrene concentration, which can be observed by a decrease in the I'/I ratio. Again, the collision rate increases with increasing temperature until the main transition temperature, t_t, is reached and pyrene is now more diluted in the completely fluidized membrane. Above t_t, I'/I increases again with temperature. At very low pyrene concentrations (Fig. 1D), excessive dilution already occurs at the pretransition, leading to a minimal

value of I'/I, which is then determined by the long-wavelength branch of the monomer emission at the wavelength of the otherwise observable maximum of the excimer band. This value increases steadily above t_p.

To provide further evidence for this interpretation, we used a pyrene concentration of which both pre- and main transitions were clearly observable (Fig. 3). Cholesterol, which is known to disturb preferentially the $P_{\beta'}$ phase, causes a decrease in the pretransition step at concentrations up to 30 mmol cholesterol per mol lipid, whereas the main transition step is only slightly broadened. This finding is consistent with the electron microscopic study of Sackmann et al. [17] who reported a maximal incorporation of 60 mmol cholesterol per mol lipid into defect regions of the pure lipid. The defect pattern above and below the pretransition became identical at 60 mmol cholesterol, which means that the pretransition vanishes as is observed in the present excimer study (Fig. 3, curve d). We conclude that defect structures are able to take up solutes which may diffuse along the defect lines.

Clear evidence for the uptake of solutes by the defect pattern is obtained from the determination of partition coefficients of chlorpromazine between the lipid and the aqueous phase. Chlorpromazine, an amphipathic drug, is known to dissolve well in fluid lipids but poorly in the crystalline state [10]. Here we focussed on the $P_{\beta'}$ phase. Values given in Fig. 4 are mean values of at least five determinations at a given temperature with an average deviation of not more than $\pm 5\%$.

We observed a significant increase in the partition coefficient at the pretransition temperature, from $k_p \approx 300$ to $k_p \approx 750$. Above the lipid phase transition the value is about 3200 ± 300. Again, these results may be explained by the consideration of a fluid defect pattern in the $P_{\beta'}$ phase. If 20% of the lipid in the $P_{\beta'}$ phase is present in defect structures, as was reported from the photobleaching experiments, and if we assume the same solubility of chlorpromazine in either fluid lipid, we have to expect a k_p value around 600–700, which fits the experimental result. Then, below the pretransition temperature the k_p value has to be lower by about a factor of 3.

Low amounts of cholesterol should occupy the defects and therefore inhibit the amphipath incorporation. The partition coefficient, k_p, decreases from about 750 to about 500 at 35°C. At high concentrations of cholesterol, the incorporation of chlorpromazine is possible only above the main transition temperature. We conclude that the defect structures of the $P_{\beta'}$ phase and the L_β phase behave like fluidized lipids within a crystalline matrix.

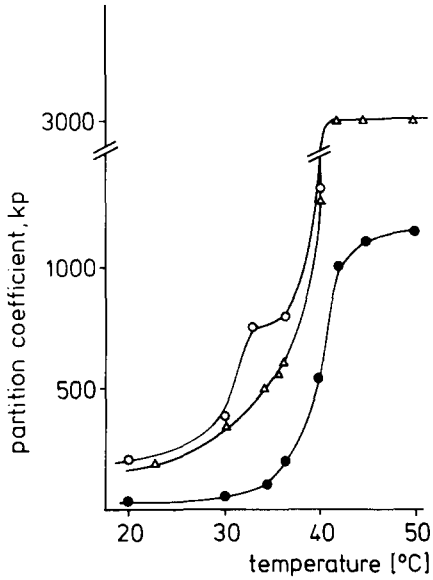

Fig. 4. Partition coefficients of chlorpromazine in dipalmitoylphosphatidylcholine vesicles as function of temperature in the absence (○) and in the presence of 30 mmol (△) and 200 mmol (●) cholesterol with respect to the lipid. All values are means of at least five determinations and the deviations are better than $\pm 5\%$ below the main transition temperature. Note the break in the ordinate at $k_p > 1500$. Values of pure DPPC membranes at $t > t_t$ are 3200 ± 300 [10] and are not significantly different from values in the presence of 30 mmol cholesterol with k_p values of $k_p = 3000 \pm 300$ at $t > t_t$. Values between the pre- and the main transition temperature are significantly different.

The last point to discuss is the mechanism of the diffusion in the postulated channel structure. In earlier work by ourselves [4,5] and others [6,7], evidence was given for the applicability of the free volume model to describe diffusion processes in fluid membranes. The question arises of the applicability of this model to diffusion in fluidized defect structures of the $P_{\beta'}$ phase or even the L_β phase. Direct proof is lacking; however, the pressure dependence shown in Fig. 1 fits the model.

Firstly we may compare the dt/dP values determined at the pre- (t_p) and the main transition temperature (t_t). The value obtained at the pretransition, $dt_p/dP = 22$ K/kbar, is lower compared to the value at the main transition $dt_t/dP = 28.6$ K/kbar. Using the Clausius-Clapeyron equation it is possible to calculate the volume change, ΔV, accompanying the phase transition: $\Delta V = (dt/dP) \cdot (\Delta H/T)$ where dt is the shift of the pre- or main transition temperature, t, with pressure. Using the enthalpy values $\Delta H_t = 35$ kJ/mol and $\Delta H_p = 7.5$ kJ/mol from Mabrey and Sturtevant [14] and the corresponding transition temperature $T_t = 314$ K and $T_p = 306$ K, we obtain $\Delta V_p = 5.4$ ml/mol and $\Delta V_t = 32$ ml/mol. These values are close to the values determined by Nagle and Wilkinson [18].

Theoretical values of ΔV_p calculated by Scott [19] on the basis of rotating hard rods are much lower and clearly show that additional degrees of freedom have to be available beside pure rotational freedom. It is not proven but it is tempting to speculate that the high transition volume of the lipid pretransition is correlated with the appearance of fluidized defect structures within the crystalline lipid phase.

Moreover, the pressure depression of the excimer/monomer intensity ratio at temperatures between the pre- and the main transition temperature is well comparable to the depression in the fluid phase. Such an effect cannot be explained by probes embedded in a crystalline matrix that hinders or even freezes free diffusion. The results fit the model of solutes that diffuse freely within defect structures which behave like a fluid lipid phase for which the free volume model may be applied. Unfortunately, it is impossible to calculate diffusion coefficients within the defects from the excimer technique because of the unknown probe concentration and we have to acquiesce in the qualitative arguments using this method.

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