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ON THE PHASE TRANSITION KINETICS OF PHOSPHOLIPID BILAYERS

RELAXATION EXPERIMENTS WITH DETECTION OF FLUORESCENCE ANISOTROPY

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Relaxation experiments were performed on vesicles of dimyristoylphosphatidylcholine in the lipid phase transition region by means of a Joule heating temperature jump technique. The time course of fluorescence anisotropy of the dye 1,6-diphenyl-1,3,5-hexatriene (DPH), incorporated in the bilayer, was observed. Since the dye always exhibits stationary anisotropy in the time range of observation, its anisotropy represents the order of the bilayer during the entire course of the experiment. Two relaxation processes were detected within the 1–100 ms range with maximal time constants at the midpoint of transition. At least one process was faster than the temperature jump dead time. The relaxation times, especially the maximal relaxation times, depend strongly on the bilayer curvature: larger vesicles imply larger time constants. This observation can explain differences between kinetic results of different laboratories. A comparison between the kinetic findings by means of three different dyes located at different sites along the lipid molecules suggests that the slower steps of the lipid phase transition mechanism involve the entire lipid molecule rather than individual parts of the molecule. The results of this present contribution reconfirm the recently published phase transition mechanism which postulates a series of steps of which the first, the kink formation, is fast and noncooperative and the following ones, representing the expansion of the aggregates, are slower and cooperative.

Introduction

Although the knowledge on the structure of phospholipid bilayers has reached a state of comprehensive understanding on the molecular level, a uniform picture of the dynamics of intramembrane processes appears to be missing so far. However, an ample variety of physico-chemical techniques has been applied to the investigation of the dynamics of individual portions of lipid molecules, of whole lipid molecules and of their aggregates. The attention of these investigations has been focused mainly on properties of the one component lipid bilayer which exhibits a solid-fluid (gel-liquid crystalline) phase transition under convenient thermodynamic conditions.

A concise summary is to briefly classify the observable processes and substantiate this classification by means of the clearest experimental investigations on the topic. This will also render the background for the kinetic experiments presented in this paper.

Two classes of dynamic processes should be

Abbreviation DPH, 1,6-diphenyl-1,3,5-hexatriene

differentiated in the temperature interval of the lipid phase transition:

(a) Processes with unimolecular time constants which vary according to an Arrhenius type of temperature dependence and/or change magnitude with a magnitude-temperature profile generally recognized as a transition curve. These processes represent changes in the mobility of individual molecules or their parts as influenced by the state of their environment. They be called (noncooperative) isomeric changes.

(b) Processes with unimolecular time constants which exhibit a pronounced maximum at the transition midpoint temperature and fall off rapidly as temperature is increased or decreased from the midpoint of transition. These properties represent a change of the 'lattice type' aggregate in which the individual molecules are embedded. And these processes be called cooperative because they involve the concerted action of many molecules which implies that in the phase transition region they are much slower than the noncooperative processes. And in fact the noncooperative ones occur in the picosecond to nanosecond range whereas the cooperative ones were measured in the millisecond to second range.

Very specific and fast methods were applied for the observation of the noncooperative intramembrane processes. These allowed not only the investigation of individual molecules, but of characteristic portions of molecules. The dynamic behaviour of the zwitterionic phosphatidylcholine head group was characterized by dielectric dispersion and absorption. Relaxation time constants between 1 and 150 ns were obtained [1–6]. Information about the rate of rotational motion of lipid chain segments was provided by the application of a pulsed NMR-technique on specificly deuterated lipids. Here correlation time constants between 10 and 100 ps were obtained [7]. In addition, the time constants for individual chain segments could be correlated with order parameters from deuterium quadrupole splittings for these segments.

Somewhat more indirect, but nonetheless quantitative information on order and rotational mobility of lipid chains is derived from steady-state anisotropy decay of fluorescent dye labels [8,9]. In the experiment order and mobility are determined for the probe molecules alone. But for the dye 1,6-diphenyl-1,3,5-hexatriene (DPH) which partitions equally between the solid and the fluid phases of the lipid bilayer [10] it was shown recently by comparison with deuterium NMR data that the probe order parameter correlates well with the order parameters from deuterium quadrupole splittings of the tenth and the twelfth chain segment in dipalmitoylphosphatidylcholine bilayers [8]. Time constants of the fluorescence anisotropy decay were determined between 1 and 10 ns [11–14]. But also for a different label (2-anilinonaphthalene) which is supposed to be similarly incorporated, a practically identical decay constant has been observed [15]. The anisotropy decay constants and the time constants from the dielectric methods can be compared with the results of much less specific methods like ultrasound absorption [16] and temperature jump relaxation with turbidity detection [17]. For both investigations the observed effects were attributed to the formation of rotational isomers in the lipid chains. As mentioned above the most important common feature for all of these fast processes in the bilayers is a weak, probably Arrhenius type temperature dependence and in most experiments the reported motions were faster above than below the phase transition temperature. After these mostly very specific approaches, the fast noncooperative effects could be considered as neatly characterized.

This is not the case yet for the slower cooperative effects for whose investigation only chemical relaxation methods with mainly unspecific detection (turbidity) were applied [18–20]. A full appreciation of literature will be given in the discussion. However, the comprehensive knowledge about the DPH label, as outlined above, provides a handy tool for a more quantitative investigation of the kinetics of the cooperative processes involved in the lipid phase transition. Just before completion of this manuscript Strehlow and Jahnig [21] published their work on the kinetics of the electrostatically triggered phase transition making use of the DPH label. For this present report the label was applied to the phase transition as a means for detection of fluorescence anisotropy changes due to a temperature jump method.

Experimental

Preparation of vesicles

The lipid β,γ-L-dimyristoyl-α-phosphatidylcholine and the fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Fluka, Switzerland, and used without further purification. Vesicles were prepared by injecting an ethanolic lipid solution into the buffer at 35°C [22]. Buffer conditions were 0.01 M Tris-HCl (pH 7.5), 0.1 M NaCl and 1 mM NaN_3. After a dialysis against pure buffer of at least 8 h the vesicle solution was heated to 50–60°C and 2 mM DPH in tetrahydrofuran (Fluka, p.a.) were added under heavy stirring. The volumes were appropriately chosen to achieve the desired lipid/label ratio. Subsequently the solution was kept at the mentioned temperature for 30 min during which the added tetrahydrofuran was to evaporate. The kinetic experiments were performed immediately thereafter.

Temperature jump technique

The technical design of the temperature-jump apparatus resembles very closely the one which was published by Rigler et al. [23]. By means of a high voltage power supply (Instrumenten-Gesellschaft) a 50 nF capacitor is charged to 10 kV and discharged through the sample cell containing the observation chamber with the aqueous electrolytic solution under investigation. This discharge results in a temperature increase of 0.7 K within 5 μs at our conditions. At the present set-up this is the minimum temperature rise achievable. It is limited by the quality of the spark in the spark gap and therefore by the capacitor voltage. The optical arrangement is mounted on a mechanically insulated marble table. It consists of a mercury/xenon lamp (200 W), a double monochromator set at 365 nm (Schoeffel), one polarizing prism in the exciting light beam, and one filter for 400 nm cut-off wavelength (Schott, Mainz) and one polarizing coat filter (3 M Brand Polacoat) at both 90° emission angles. The emitted light is detected by EMI Q 9558 A photomultipliers. The two photomultipliers are equilibrated to identical output voltage for identical light intensity. This was done by choosing the polarizing planes of the polarizer and the two analysers as follows: polarizer horizontal, one analyser vertical, one horizontal. The final set-up for the observation of anisotropy was reached by turning the polarizer vertical. Because turning of the polarizer influences the intensity of the exciting light beam which is partially polarized already by the monochromator, the equilibration was performed with an attenuating gray filter in the exciting light beam. In order to achieve a good signal to noise ratio the total light intensity had to be chosen relatively high. This has the consequence that the measured anisotropy turns out lower than in other, static experiments [24]. Another source for the relatively low anisotropy can be seen in the fact that the polarizing prism could not be aligned in the exciting light beam so that 100% of the light from the monochromator was polarized. This was due to the fact that the monochromator slits had to be opened wide for the sake of light intensity.

The temperature was controlled inside the ground electrode of the *T*-jump cell to an accuracy of ±0.1 K. The temperature was scanned from above to below the phase transition temperature T_m. Immediately before each temperature jump the equilibrium anisotropy was determined and thus simultaneously with the kinetic experiment the phase transition curve was obtained.

Results

As outlined in the introduction, the dye molecule DPH was chosen as a probe for the lipid chain order as a function of temperature because its properties in the hydrophobic environment of the membrane has been well characterized. It is plausibly assumed that DPH indicates the order conditions of the membrane at the depth of about the 10th or 12th carbon atom of the lipid chain as counted from the backbone towards the end of the chain. During the temperature jump experiment the DPH molecule always exists in a steady state since fluorescence life time and anisotropy decay time lie around 10 and 1 ns, respectively [14,11], whereas the heating time of the temperature jump technique and thus its dead time lie at about 5 μs. Therefore the observed anisotropy r is indeed to be read as $\bar{r}$, the commonly used symbol for steady-state anisotropy [8,9].

The kinetic experiments were performed on

vesicles of different sizes which were prepared according to the data of Ref. 22. The reliability of the formation of predetermined vesicle sizes appeared to be unsatisfactory, however. Therefore it was preferrable to utilize the midpoint slope of the transition curve as an always automatically obtained equivalent to the radius. In the investigated range of vesicle sizes midpoint slope and radius are linked by a linear proportionality as could be shown recently [25]. According to Ref. 22 the vesicle radii varied between 20 nm and 60 nm. This procedure also offers the possibility for a direct comparison between the kinetics from lipid aggregates which were prepared in different laboratories without determination of vesicle sizes, as demonstrated in Fig. 4. The midpoint slope of the transition curves was determined by transforming the measured anisotropy $\bar{r}$ into the degree of transition

$$\theta = \frac{\bar{r} - \bar{r}_f}{\bar{r}_s - \bar{r}_f} \tag{1}$$

where $\bar{r}_s$ and $\bar{r}_f$ are the limiting values of the solid and fluid membrane states, respectively. Thereby it is assumed that the temperature dependence of $\bar{r}_s$ and $\bar{r}_f$ can be approximated as a linear function. With the anisotropy as a function of temperature, the midpoint slope $(\partial\theta/\partial T)_m$ could be determined. This procedure was performed for each set of kinetic experiments.

The relaxation decay curves could be satisfactorily fitted with a sum of two exponential functions plus a term which represents a very fast decay of which only the amplitude is evaluated because its time constant is smaller than the temperature rise time or the time constant of the electronics. Two original relaxation curves in a linear amplitude versus logarithmic time mode are depicted in Fig. 1. This mode of plotting was chosen because it offers a good simultaneous presentation of widely separated processes, contrary to the log/linear or linear/linear plots. The error margins of the fitting parameters (amplitudes and time constants) are drawn in Figs. 2–4.

The temperature amplitudes are depicted in Figs. 2a and 3a for experiments on different vesicle sizes. In terms of anisotropy changes they can

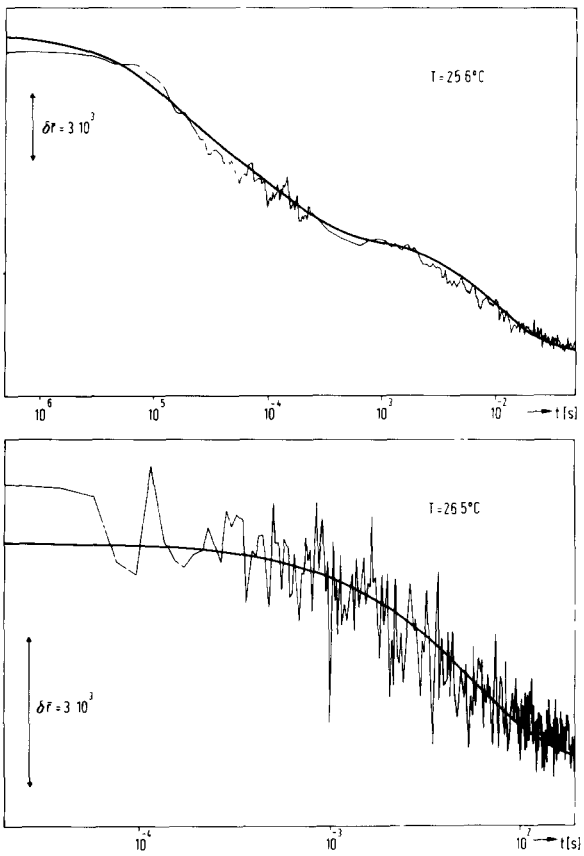


Fig 1 Temperature jump relaxation curves taken in the phase transition region of 40 nm DMPC vesicles with incorporated DPH. Fluorescence anisotropy (linear) is plotted versus time (logarithmic) Best fits of a sum of exponentials are drawn as a smooth curve (top) $T = 25.6$ °C, electronic time constant = 10 μs (bottom) $T = 26.5$ °C, electronic time constant = 10 μs.

be represented by

$$\delta\bar{r} \approx \left\{ (\bar{r}_s - \bar{r}_f)\frac{\partial\theta}{\partial T} + \frac{\partial\bar{r}_f}{\partial T} + \left(\frac{\partial\bar{r}_s}{\partial T} - \frac{\partial\bar{r}_f}{\partial T} \right)\theta \right\} \delta T \tag{2}$$

which is obtained by differentiating Eqn. 1. The first term dominates in the transition region because of the sudden change of θ with T which is described by

$$\frac{\partial\theta}{\partial T} = \Gamma \cdot \frac{\Delta H^\circ}{RT^2} \tag{3}$$

as outlined in Ref. 20. The amplitude factor Γ approaches 0 far below and above the phase tran-

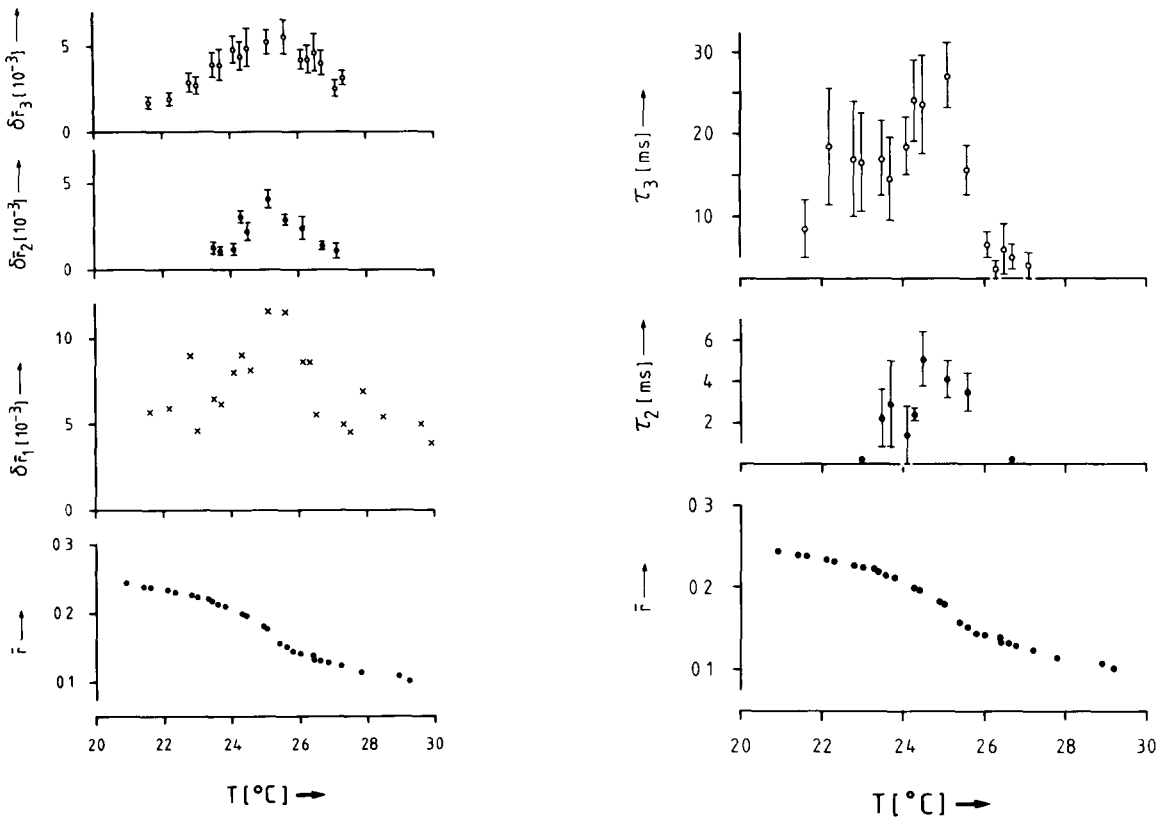


Fig. 2 Temperature jump experiments on DMPC vesicles of 40 nm radius (a) (Top three diagrams) relaxation amplitudes as a function of temperature. Physical quantity is changes of fluorescence anisotropy as described in the text The index sequence of $\delta\bar{r}_i$ corresponds to the sequence of effects with increasing relaxation times (see also text) (Bottom) Lipid phase transition curve as detected by means of fluorescence anisotropy (b) (Top and middle) Relaxation times as a function of temperature, indices correspond with those in (a) (Bottom) Phase transition curve

sition temperature T_m and passes through a maximum Γ_m at T_m. The value of $4\Gamma_m$ equals the so-called cooperative unit as could be shown in Ref. 20:

$$4\Gamma_m = \frac{\Delta H_{vH}}{\Delta H^o} \tag{4}$$

where ΔH^o is the standard transition enthalpy and ΔH_{vH} the apparent transition enthalpy which is evaluated from van't Hoff plots. Although the first term in Eqn. 2 vanishes far below and above T_m, the temperature jump induced change of anisotropy remains finite due to the last two terms of Eqn. 2.

The amplitude $\delta\bar{r}$ represents the entire transition process. Since the entire process is assumed to consist of a consecutive series of steps [17] the total amplitude $\delta\bar{r}$ must be the sum of the amplitudes of the consecutive processes:

$$\delta\bar{r} = \sum_i \delta\bar{r}_i \tag{5}$$

Increasing index i corresponds to the sequence of steps in the transition. Of course, the indeces of amplitudes and time constants of identical processes are identical as well.

The relaxation times of the corresponding processes are presented in Figs. 2b and 3b, again for experiments on different vesicle sizes. A pronounced increase of both time constants occurs in the phase transition region. Outside, the processes speed up and seem to assume limiting

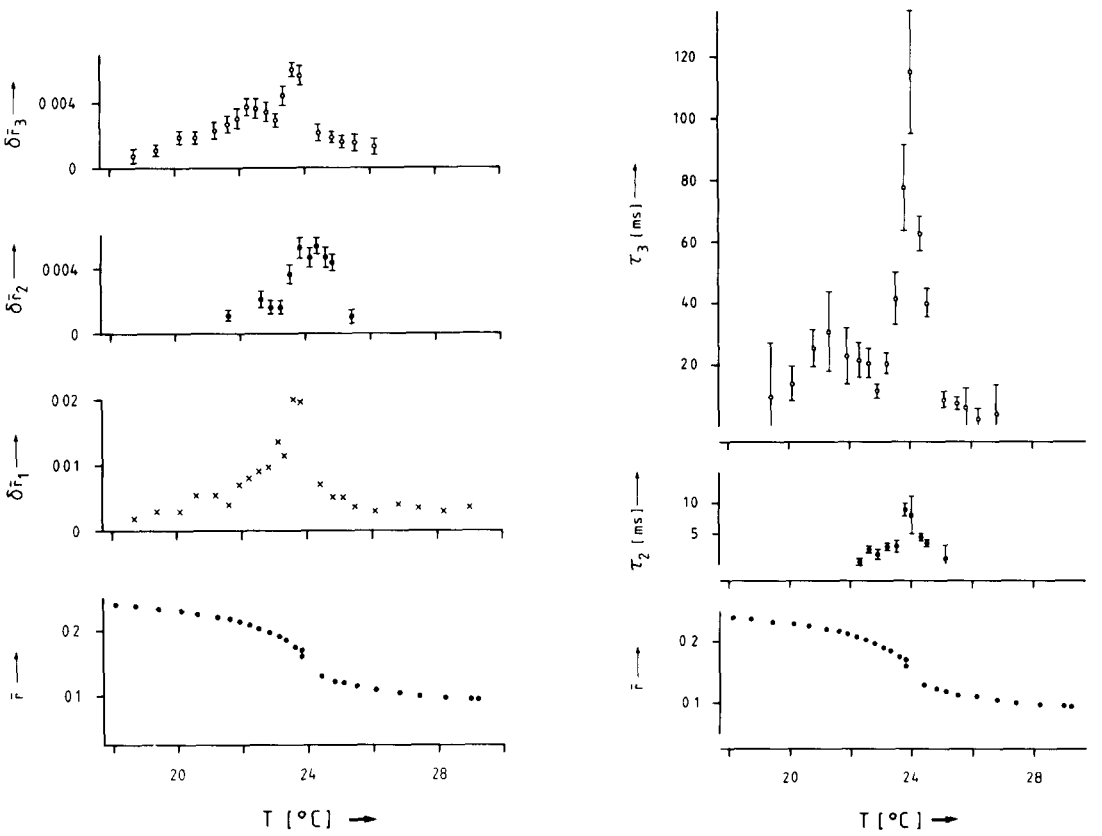
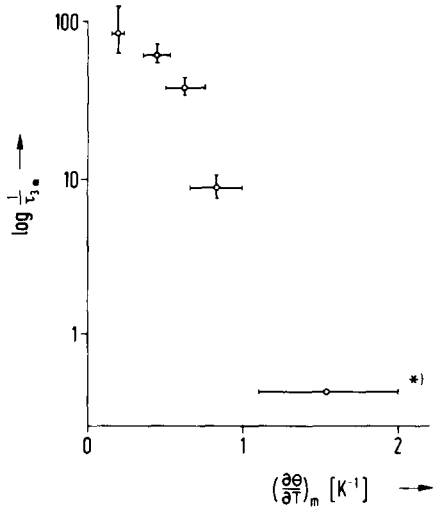


Fig. 3 Temperature jump experiments on DMPC vesicles of 60 nm radius The specifications are otherwise identical with Fig 2

values of τ which are difficult to obtain because the amplitudes of the relaxation curves vanish here. The slowing down of the relaxation processes correlates well with the transition midpoint.

It becomes evident from the presented results that an inhomogeneity of vesicle sizes must influence the relaxation time/temperature profile. An attempt to determine the vesicle sizes by means



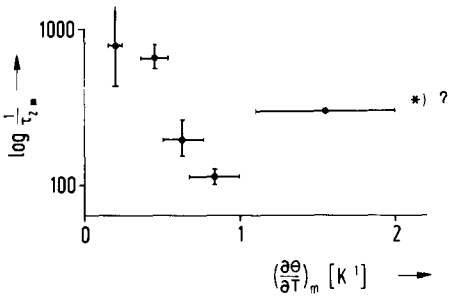
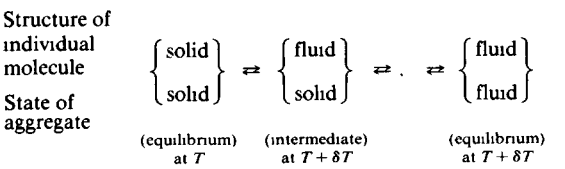


Fig 4 Temperature jump relaxation rates (logarithmic) as a function of the steepness of the lipid phase transition curve at the midpoint temperature Results were taken from the work presented in this paper and from Tsong and Kanehisa (*) [19]

of quasielastic light scattering yielded the mean sizes and reconfirmed the literature results, but failed in determining the size distribution due to a small number of channels in the correlator. However, from the characterization of the applied vesicle preparation method by Kremer et al. [22] it is known that these aggregates have a relatively uniform size. Nonetheless, since there will be a distribution of vesicle sizes in any sample prepared according to the described procedure, those vesicles having larger radii than the determined mean will contribute to an increase and a narrowing of the maxima depicted in Figs. 2 and 3, whereas those with radii smaller than the determined mean will contribute to a decrease and a broadening of the maxima.

Discussion

In a recent paper [17] we suggest a mechanism for a stepwise transition between the solid and the fluid states of the phospholipid bilayer. In brief the essentials are summarized by the following scheme:



This is to express that in a very fast first step (few nanoseconds) after installation of new equilibrium conditions the lipid molecules can assume the structure which e.g. corresponds to the new (higher) temperature. The dimensions of the lipid aggregate are not altered in this step. The necessary expansion of the aggregate to the new equilibrium dimensions proceeds in a sequence of steps. It depends strongly on intermolecular interactions and thus takes a considerably slower course. It is this final installation of the new equilibrium which was investigated in this present contribution.

As mentioned in the introduction the slowing down of these relaxation processes in the midpoint region is the physical expression for the cooperation of many molecules. The number of molecular sites in the aggregate at which simultaneous volume expansion occurs determines the rate of relaxation. This number is largest at the transition midpoint and thus the corresponding rate lowest. Some authors call the phenomenon a 'critical slowing down' [26], thereby associating it with processes at critical points in macroscopic systems.

The described properties were known from a number of publications: as temperature is varied about the phase transition temperature the relaxation time passes through a maximum at the midpoint of transition. More detailed investigations reveal that the relaxation decay is described best by the sum of two exponentials of which both time constants exhibit the mentioned characteristic [19,20,27].

A discussion remark by Teissie [27] recently raised the question which physical property causes the cooperative character of the relaxation signals. In his temperature jump experiments he observed the change of fluorescence intensity as emitted by the probe dansylphosphatidylethanolamine of which is known that the fluorescing part of the molecule is located in the glycerol backbone region of the phospholipid bilayer. The recorded kinetic results exhibit the mentioned cooperative characteristics. He concludes that the lipid phase transition should be triggered by some process in the backbone region. I believe, however, that the answer needs a different accent. Including the present experiments there are three investigations on the kinetics of lipid phase transitions in which fluorescence probes are located specificly at the three main configurational elements of the lipid molecules: 8-anilinonaphthalene 1-sulphonate in the headgroup region [28], dansylphosphatidylethanolamine in the glycerol backbone region [27] and DPH in the hydrocarbon chain region (this work) and Ref. 21. Due to differences in the aggregates (see also below) and the degree of sophistication in the kinetic analysis, there are minor differences of time constants, but all observations coincide in the result that the kinetics exhibits cooperative properties. It thus seems that the installation of an equilibrium within the transition region is a process which is experienced by individual lipid molecules as a whole, rather than by portions of these molecules. The concept of expansion of the molecular volume in the slow kinetic step, most probably at the border line of clusters, gains credibility from these results. An increased molecular volume, and simultaneously

molecular area, enables the 8-anilinonaphthalene 1-sulphonate molecule to be incorporated between the phospholipid head groups resulting in a fluorescence increase [28]; for the dansyl probe a widening of the molecular volume results in a fluorescence decrease due to a decrease of the dielectric constant in the environment [27]; and for the DPH probe it causes a decrease of anisotropy (this work).

The fact that a volume effect determines the rate of the equilibrium installation means that the former observations of an unspecific signal like turbidity is perfectly adequate because it reacts sensitively to changes of density and thus of the refractive index [19,20].

There is a certain limitation to the comparability of kinetic data presented by different authors. As pointed out in Results the relaxation times depend strongly on the type of aggregate, Fig. 3. The maximal relaxation time, e.g., increases monotonously and evidently in a nonlinear fashion with the vesicle size. Differences between various vesicle or liposome preparations should not surprise consequently. Between small vesicles and large liposomes they may easily amount to more than an order of magnitude. Recently Kaatze and Henze [6] reported a similar vesicle size dependent phenomenon: their dielectric relaxation measurements on DMPC vesicles yielded relaxation times for the rotation of the phosphatidylcholine head groups which are clearly bilayer curvature dependent. They could correlate these time constants with lipid cluster sizes implying that all molecules in one cluster move in phase.

I feel that the model on the molecular events during the course of the lipid phase transition which we recently presented [17] and which is briefly outlined above could set a frame to the various observations by means of faster and slower relaxation techniques and unite the on first sight very unequal observed properties to a comprehensive picture.

Acknowledgement

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