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ELECTROSTATIC INTERACTIONS AT CHARGED LIPID MEMBRANES

KINETICS OF THE ELECTROSTATICALLY TRIGGERED PHASE TRANSITION

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Summary

Charged lipid membranes of dimyristoylmethylphosphatidic acid were mixed rapidly in a stopped-flow cell with protons or Ca^{2+} to compensate the charges and thereby trigger the ordered-fluid phase transition. The kinetics of the transition was studied by following the time development of the fluorescence anisotropy of diphenylhexatriene. A relaxation process was observed with a characteristic time in the range 10—200 ms. By comparison with existing theories of non-equilibrium relaxation it was concluded that the relaxation process is governed by a nucleation step.

Introduction

In recent years much progress has been made in exploring the structural order of lipid membranes. It is well known that lipid order can be varied by external parameters and that the regulation of lipid order is extremely effective at the ordered-fluid phase transition. The external parameters include temperature, pressure and surface pressure. In aqueous dispersions the surface pressure can be varied electrostatically, if the lipid polar heads contain ionizable groups. The strength of the electrostatic repulsion and, consequently, the surface pressure depend on both the degree of ionization and the screening of surface

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Abbreviation DMMPA, dimyristoylmethylphosphatidic acid.

charges by counter-ions. Thus, protons and divalent cations such as Ca^{2+} as well as monovalent cations can trigger the ordered-fluid transition (for a review see Träuble [1]).

Previous studies on the kinetics of the phase transition have made use of thermal triggering, i.e. temperature-jump techniques [2–4]. Both monophasic and biphasic relaxation processes have been observed. The relaxation times were longest at the phase transition; the faster one appeared in the 10 ms range, the slower one in the range of seconds. Recently, a pressure-jump study was carried out [5] in which two relaxation times were found which increase at the phase transition; they lie, however, in the ms and 10 ms range. In all these investigations light-scattering was used to detect structural changes in the lipid dispersion. It is not yet clear, however, what type of structural change light-scattering detects.

In this paper we present the first study of the kinetics of an electrostatically triggered phase transition. In stopped-flow experiments, the concentration of protons or Ca^{2+} in a dispersion of charged lipid membranes is suddenly raised, and the lipids undergo the phase transition in the direction of higher order. Changes in lipid order are detected by means of the fluorescence anisotropy of the dye diphenylhexatriene, which was shown by one of the authors to be a quantitative measure of the lipid orientational order [6]. Within the experimental error, the observed kinetics was monophasic with a relaxation time in the range of 10—200 ms, depending on the experimental conditions. The latter were chosen in order to transfer the lipid phase into non-equilibrium states at different displacements from the equilibrium phase transition. The dependence of the relaxation time on the displacement is utilized to identify the relaxation mechanism which gives rise to the observed kinetics.

Materials and Methods

Materials. Dimyristoylphosphatidylcholine was purchased from Fluka (Buchs, Switzerland). Dimyristoylmethylphosphatidic acid (DMMPA) was obtained from this lipid by de-esterification using phospholipase D [7], and was purified by chromatography. According to thin-layer chromatography the product was pure. A ^{31}P-NMR spectrum showed two types of impurity in amounts less then 2%. Na and $CaCl_2$ (of 'suprapur' grade) were from Merck (Darmstadt, F.R.G.). Water was double-distilled. Melittin was purchased from Serva (Heidelberg, F.R.G.). The fluorescent dye diphenylhexatriene was a gift from Dr. Shinitzky of the Weizmann Institute (Rehovot, Israel).

Sample preparation. The anhydrous sodium salt of DMMPA was suspended in 0.1 M NaCl solution at pH 7 to yield a dispersion of 10^{-2} M in lipid. For the experiments with calcium the lipid was suspended in water. The dispersion was incubated for 1 h at 55°C, then sonicated for 15 min at 12°C in a Branson bath-sonicator (set at 30 W), and diluted to the desired concentration of $2 \cdot 10^{-4}$ M or $4 \cdot 10^{-4}$ M for the static or kinetic measurements, respectively. The dye was taken from a stock solution of 10^{-3} M diphenylhexatriene in tetrahydrofuran and added to a final concentration of $2 \cdot 10^{-6}$ M. The incubation time was 2 h at 55°C in order to reach equilibrium of the partitioning between the water and the lipid phase. These dispersions were stored and only adjusted

to the desired pH value using 0.1 N HCl or 0.1 N NaOH immediately before the measurement. Over a period of 2 h no degradation was observed under our experimental conditions. This method of sample preparation assured reproducibility.

DMMPA and melittin were mixed in chloroform at a molar ratio of 100 : 1, the solvent was removed under a stream of argon, and the sample was dried in high vacuum, then treated as above. Traces of phospholipase activity in melittin were inhibited by the addition of 10^{-3} M EDTA.

Fluorescence stopped-flow measurements. Fluorescence anisotropy was measured in a combined temperature-jump/stopped-flow apparatus as described by Rigler et al. [8] and Jovin [9]. Two photomultipliers were used to detect the intensities of the fluorescence light polarized parallel ($I^{\parallel}$) and perpendicular ($I^{\perp}$) with respect to the polarization of the excitating light. The fluorescence anisotropy is defined as

$$r_S = \frac{I^{\parallel} - I^{\perp}}{I^{\parallel} + 2I^{\perp}} . \tag{1}$$

The fluorescence anisotropy of diphenylhexatriene is a measure of the lipid order in the membrane. It has been shown [6] that r_S is closely related to the orientational order parameter S of the lipid hydrocarbon chains known from deuterium magnetic resonance, and to a good approximation the following relation holds

$$r_S = \tfrac{2}{45}(1 + 8S^2) . \tag{2}$$

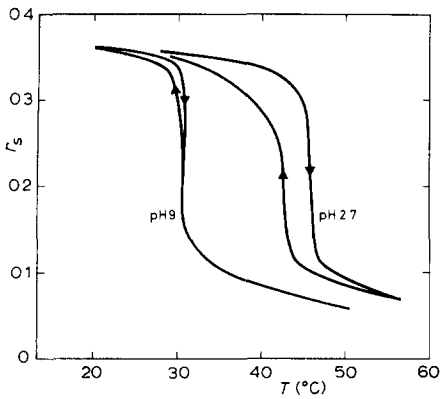
In parallel experiments it was shown that qualitatively the same kinetic results are obtained using the fluorescence intensity of *N*-phenylnaphthylamine for detection. Because in this case the kinetics of the phase transition is superimposed upon the kinetics of the dye partitioning between water and lipid, this technique was not pursued further.

The dead time of the instrument was determined to be 9 ms. The mixing process was shown to be faster than the dead time by following the absorption at 625 nm of bromocresol green, a pH-sensitive dye added to the dispersion; immediately after triggering the mixing process, the absorption indicated the value of the final pH. We note that electron micrographs of our mildly sonicated DMMPA dispersions showed neither small vesicles nor onion-like structures, but sheet-like structures, so that all lipid head groups are probably accessible to protons without the need to permeate the bilayer.

Results

Static measurements

The fluorescence anisotropy of diphenylhexatriene in DMMPA liposomes was recorded as a function of temperature for different pH values. Two examples are shown in Fig. 1. The sharpness of the transition is typical for large liposomes obtained by mild sonication, in contrast to small, highly curved vesicles obtained after extended sonication. At pH 2.7, where the lipid polar heads are uncharged, a pronounced hysteresis is observed, which is absent at pH 9, where the lipid molecules are negatively charged. The transition temperatures T_t at



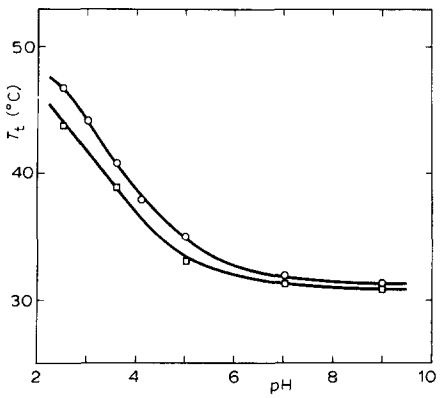


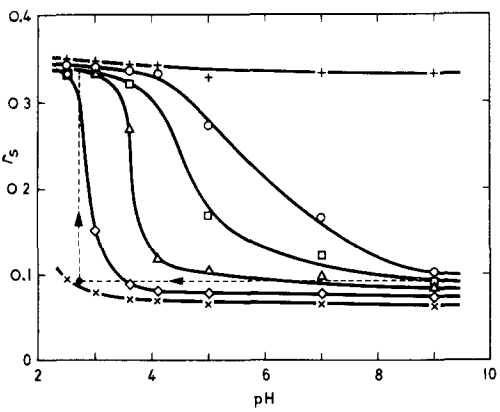
Fig. 1. Temperature dependence of the diphenylhexatriene fluorescence anisotropy at two different pH values. $2 \cdot 10^{-4}$ M DMMPA/0.1 M NaCl.

Fig. 2. Variation of the phase transition temperature with pH for increasing (○) and decreasing (□) temperature.

different pH values are shown in Fig. 2. They agree with earlier results obtained from fluorescence measurements [10] and calorimetry [11] for the same lipid. Data from experiments analoguous to those in Fig. 1 are compiled in Fig. 3 to show the pH dependence of the anisotropy at various temperatures. According to Eqn. 2 the plots in Figs. 1 and 3 reflect the variation of the orientational order parameter of the lipid chains.

Divalent metal ions such as Ca^{2+} can also be used to neutralize the lipid charges and are shown in Fig. 4 to have the same effect as protons (cf. Ref. 1).

The incorporation of small amounts of the polypeptid mellitin (mellitin : lipid mol ratio, 1 : 100) had no effect on the temperature dependence of the



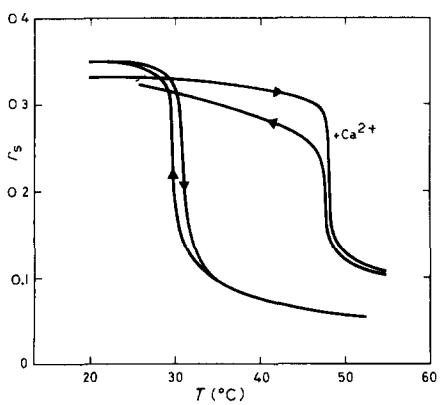


Fig. 3. pH dependence of the diphenylhexatriene fluorescence anisotropy at different temperatures, constructed from data as in Fig. 1. The dashed lines show the course of a stopped-flow experiment, with the non-equilibrium state (●). +, 30°C; ○, 35°C; □, 37°C, △, 40°C; ◇, 45°C; X, 50°C.

Fig. 4. Temperature-dependence of the diphenylhexatriene fluorescence anisotropy without and with Ca^{2+} at pH 9. $2 \cdot 10^{-4}$ M DMMPA/10^{-3} M NaCl, Ca^{2+} added to a final concentration of 10^{-3} M $CaCl_2$ corresponding to a molar ratio Ca^{2+}:DMMPA of 5.

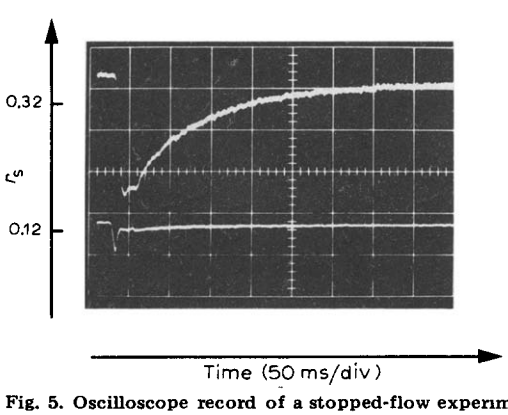
fluorescence anisotropy, except for the disappearance of the hysteresis at low pH values.

Kinetic measurements

The kinetics of transitions between fluid and ordered states of the lipid phase was investigated by measuring the time dependence of the fluorescence anisotropy in a stopped-flow cell. In the first series of experiments a DMMPA dispersion of pH 9 and an aqueous solution of pH 2.35 at the same ionic strength were mixed to yield a final pH of 2.7. Since the establishment of the final pH was shown to be faster than the dead time of the instrument, the much slower relaxation process which we observed in the 10—200 ms range corresponds to a structural transition in the lipid membrane. As stated previously, the fluorescence anisotropy of diphenylhexatriene detects changes in the orientational order of the lipid chains, so we observe a transition from a non-equilibrium fluid state to an equilibrium ordered state, as indicated in Fig. 3.

A typical example of the time course of the fluorescence anisotropy in the case of the proton-triggered phase transition is shown in Fig. 5. The amplitude of the process corresponds to the transition between the fluid and the ordered phase. Taking the experimental uncertainty into consideration, the time course can be approximated well by a single exponential, with the relaxation time τ. Träuble [2] also evaluated his kinetic data in terms of a single relaxation time, whereas Tsong and Kanehisa [3,4] and also Gruenewald et al. [5] used two. The pronounced temperature dependence of the relaxation time we found is shown in Fig. 6. With increasing temperature the phase transition proceeds more slowly. If this temperature variation is described by an Arrhenius expression the formal activation energy would be negative and equal to --60 kcal/mol, however, the significance of such a negative value is unclear.

As for the static experiments, the phase transition can also be observed by



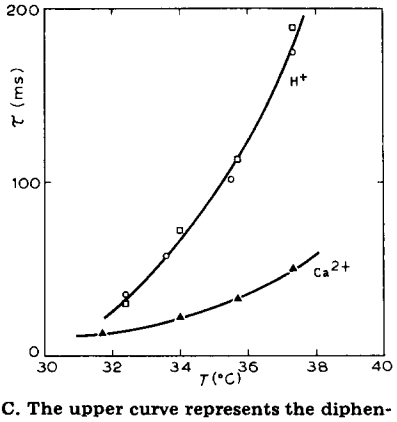


Fig. 5. Oscilloscope record of a stopped-flow experiment at 34° C. The upper curve represents the diphenylhexatriene fluorescence anisotropy, the lower curve the intensity.

Fig. 6. Relaxation time as a function of temperature for proton (○, □) and calcium (▲) triggering. Starting solution in syringe 1: $4 \cdot 10^{-4}$ M DMMPA/0.1 M NaCl, pH 9, starting solution in syringe 2. 0.1 M NaCl, pH 2.35 for proton triggering, and pH 9 and $2 \cdot 10^{-3}$ M $CaCl_2$ for calcium triggering. Proton triggering was studied without (○) and with (□) 10^{-3} M EDTA in both syringes.

addition of Ca^{2+}. In this case the time course of the fluorescence anisotropy is described less well by a single relaxation process. Nevertheless, we evaluated the data for one relaxation time which is included in Fig. 6. It is smaller by a factor of about 4 compared with the proton triggering, again in agreement with earlier observations of Träuble [2]. A similar result was obtained when the phase transition was triggered by protons in the presence of 1 mol% melittin. Melittin accelerates the phase transition by a factor of about 8.

Discussion

The static properties of lipid membranes are understood rather well, especially the ordered-fluid phase transition. In the theories which describe the experimental results best, the phase transition originates in the orientational degree of freedom of the lipid hydrocarbon chains and, therefore, is of first-order [12—14]. This means that the orientational order of the lipid chains and, in consequence, also their positional and conformational order change abruptly at the phase transition. A pronounced variation of the lipid order in the vicinity of the first-order transition, which is reminiscent of a continuous or second-order transition, makes the phase transition appear broadened to some extent.

It is important to note that since the diphenylhexatriene fluorescence anisotropy measured in our experiments detects the lipid chain orientational order (Eqn 2) it detects the basic type of lipid order the change of which gives rise to the phase transition.

The influence of surface charges on the phase transition has been studied in detail [10,15,16]. The decrease of the transition temperature caused by surface charges was found to originate from two superimposed effects: (i) With the abrupt lateral expansion at the phase transition, the energy of the electrostatic field is decreased, thus facilitating the phase transition and lowering the transition temperature; and (ii) the electrostatic repulsion of the polar heads gives rise to a tilt of the hydrocarbon chains, thereby making the chains effectively shorter with a lower transition temperature [16]. Theoretically one can show that the transition temperature T_t varies in proportion to the electrostatic surface pressure, which itself is proportional to the degree of dissociation, α [15]. If α is determined from the bulk pH by use of a relation which was derived earlier within the framework of the Gouy-Chapman theory and is plotted in Fig. 7, our experimental results for T_t (pH) indeed obey this linearity between the transition temprature and the degree of dissociation, as shown in Fig. 8.

The kinetics of the ordered-fluid phase transition is less well understood. This is a consequence of the fact that the kinetics of first-order transitions, in general, is understood less than their statics. If by a rapid change of an external variable a system with a first-order transition is transferred to a non-equilibrium state, one normally distinguishes two different mechanisms of relaxation into the new equilibrium state, according to whether the non-equilibrium state is unstable or metastable [17—19]. In the first case, the state is unstable against 'homophase' fluctuations: Wave-like fluctuations of the critical order with wave numbers below a certain threshold tend to increase with time, their wave numbers simultaneously decrease until the new homogeneous equilibrium state is established. This mechanism is often referred to as spinodal decomposition and

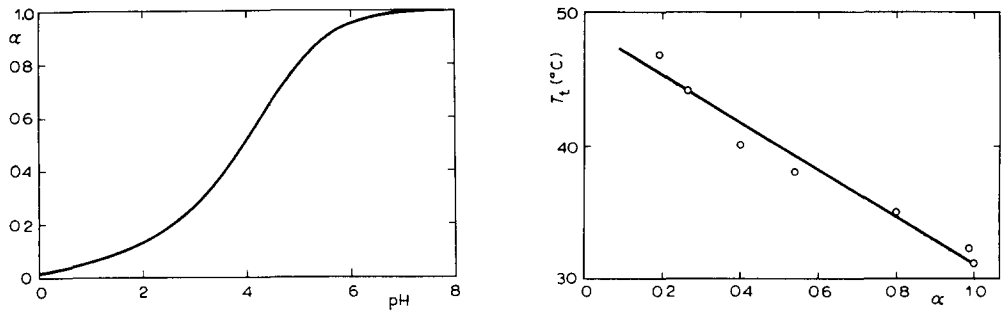


Fig. 7. pH dependence of the degree of dissociation, α, of DMMPA with a $pK_0 = 1.75$ (in the absence of surface electrostatic effects) for 0.1 M salt concentration, calculated within the Gouy-Chapman theory [15].

Fig. 8. Phase transition temperature, from Fig. 2, for increasing temperature as a function of the degree of dissociation, from Fig. 7.

described by the classical Ginzburg-Landau and Cahn-Hilliard theories [20]. In the second case, the far-from-equilibrium state is unstable against 'heterophase' fluctuations: Nuclei (also called clusters or droplets) of the equilibrium type of order above a certain size tend to grow with time, until the new equilibrium phase is established. The earliest description of this mechanism goes back to Becker and Döring [21] who investigated the condensation of a gas. Later refinements and generalizations have been worked out by several groups. Binder and Stauffer [18] and Langer [19] pointed out, both theoretically and by computer experiments, that the two relaxation mechanisms distinguished above do not occur as clearly separated processes, but are extreme cases with a continuous transition in between. A detailed application of the nucleation mechanism to membranes was given by Adam [22] in a study of nerve excitation and by Kanehisa and Tsong [23] in their study of the lipid ordered-fluid phase transition. In summary, the more recent theories offer no substantial improvements compared to the classical theories. Therefore, in the following section we try to interpret our results within the framework of the classical theories.

The relaxation time τ based on the 'homophase' flucutation mechanism was derived by Binder [17] within the Ginzburg-Landau theory as

$$\tau \sim |x - x_t|^{-1/2} \tag{3}$$

where x is the external parameter, the variation of which elicits the phase transition and $x - x_t$ is the displacement of the non-equilibrium state (x) from the phase transition point (x_t) *. This relation shows a power-law behavior characteristic of 'homophase' fluctuation phenomena. In our experiments the phase transition was triggered by the surface pressure which is proportional to the degree of dissociation so that we have $x \equiv \alpha$ and

$$\tau \sim (\alpha_t - \alpha)^{-1/2} \tag{3a}$$

* More exactly, x_t would be replaced by the value for the limit of metastability x^*. For far-from-equilibrium situations, $x >> x^* - x_t$ and the difference between x^* and x_t can be neglected.

The relaxation time for the 'heterophase' fluctuation mechanism is dependent on the spatial dimension d of the system [18], and we discuss the general case of arbitrary d. Nucleation is an activation process and the rate $k = 1/\tau$ follows an Arrhenius relation

$$k \sim \exp[-\Delta G/kT] \tag{4}$$

The activation energy ΔG is the sum of a volume term representing the energy difference between the droplet phase and the bulk phase, and a surface term representing the interfacial energy between the two phases. If R is the radius of a spherical droplet and σ a normalized surface tension we may write [18,22]

$$\Delta G = -R^d(x_t - x) + R^{d-1}\sigma \tag{5}$$

The volume term is negative and favors the creation of droplets of the new phase, more pronouncedly the further the non-equilibrium state is from equilibrium. The surface term is positive and acts against the creation of droplets. The two terms vary in different ways with the droplet radius. For large R the volume term dominates and for small R the surface term, so that ΔG is maximal at some value R_c. This critical value has to be exceeded for the droplet to grow and, therefore, in the Arrhenius formula we have to insert the corresponding energy barrier $\Delta G(R_c)$. Differentiation of Eqn. 5 yields

$$R_c = \frac{d-1}{d} \cdot \frac{\sigma}{x_t - x} \tag{6}$$

and insertion of this value into Eqn. 5 leads to

$$\Delta G(R_c) \sim (x_t - x)^{1-d} \tag{7}$$

so that Eqn. 4 becomes

$$k \sim \exp\left[-\frac{\text{constant}}{(x_t - x)^{d-1}}\right] \tag{8}$$

For the case of a three-dimensional system we get the result $k \sim \exp[-\text{constant}/(x_t - x)^2]$, which corresponds to the classical Becker-Döring formula if $x_t - x$ is the supersaturation [21]. For our case of a two-dimensional system we obtain the result

$$\tau \sim \exp\left[\frac{\text{const.}}{\alpha_t - \alpha}\right] \tag{8a}$$

which is analoguous to the result of Adam [22]. The Arrhenius-type temperature-dependence is replaced by the dependence on the displacement of the non-equilibrium state from the phase transition, $\alpha_t - \alpha$, thus resolving the problem of a negative activation energy.

In order to interpret our experimental result for τ (Fig. 6) in terms of one of the two relaxation mechanisms, Eqns. 3a or 8a, we have to transform $\tau(T)$ into $\tau(\alpha_t - \alpha)$. To do this we note that, according to the linearity shown in Fig. 8, different temperatures T correspond to different values of α_t, whereas the degree of dissociation of the non-equilibrium state in our experiments is fixed at $\alpha = 0.21$, according to the final pH of 2.7 and the relation between α and the

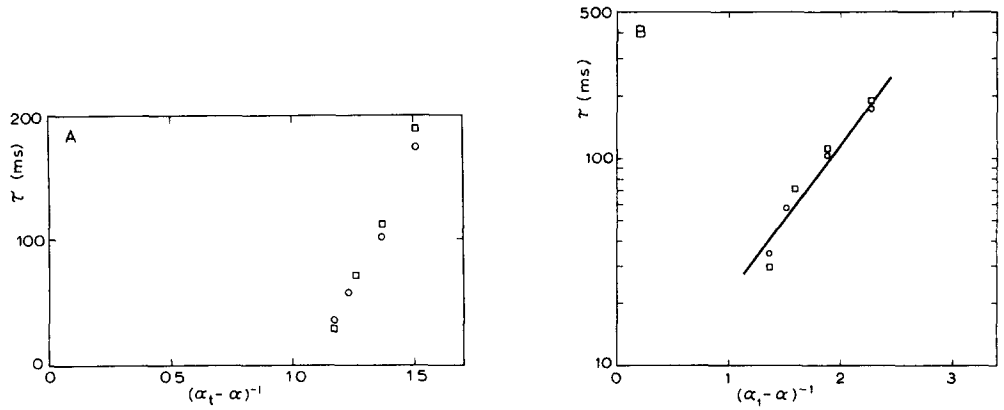


Fig. 9. Plots of the relaxation time versus displacement of the non-equilibrium state from the phase transition point to test the 'homophase' (A) and 'heterophase' (B) fluctuation mechanism.

pH given in Fig. 7. The functional dependence $\tau(\alpha_t - \alpha)$ derived in this way is plotted in Fig. 9. If the actual relaxation mechanism is of the 'homophase' fluctuation type, the plot in Fig. 9A should yield a straight line through the origin according to Eqn. 3a. The data points do not exhibit this behavior; if they are regarded as lying on a straight line, this line certainly does not pass through the origin. So the relaxation mechanism is not of the 'homophase' fluctuation type. In the case of the 'heterophase' fluctuation mechanism the plot in Fig. 9B should yield a straight line corresponding to Eqn. 8a. Obviously, the data points do show this behavior and we conclude that the relaxation mechanism we observe is of the 'heterophase' fluctuation type.

Conclusion

We have shown that the kinetics of the ordered-fluid phase transition is governed by a nucleation process. This is in agreement with the theory of Kanehisa and Tsong [23], who, however, did not test their theory quantitatively by comparison with experimental results. It is clear furthermore that this interpretation allows for an understanding of the observed influence of melittin. Due to the lipid-protein interactions, melittin molecules in the fluid lipid phase increase the order of the lipid molecules in their neighborhood [6]. Therefore, they act as nuclei for the formation of the ordered phase, thus accelerating the phase transition (heterogeneous nucleation). The finding that Ca^{2+} evoke more rapid kinetics may be explained in a similar way. Ca^{2+} bridges two lipid polar heads and thus has a clustering effect. This may facilitate the nucleation process.

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