Biochimica et Biophysica Acta, 597 (1980) 41—52

BBA 78653

KINETIC INVESTIGATIONS ON THE PHASE TRANSITION OF PHOSPHOLIPID BILAYERS

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(Received July 11th, 1979)

Key words: Phase transition; Phospholipid bilayer; Pressure-jump kinetics; Cooperative unit

Summary

Pressure-jump experiments were performed on vesicles and liposomes of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine following the time course of solution turbidity. For both lipids two relaxation effects were evaluated the time constants of which exhibit clear maxima at the midpoint of the phase transition. The time constants lie for vesicles in the 100 μs and 1 ms ranges and for liposomes in the 1 ms and 10 ms ranges. The processes are slightly faster for dimyristoyl phosphatidylcholine than for dipalmitoyl phosphatidylcholine. All relaxation times are concentration-independent. The time constant and amplitude behaviours indicate that all processes are cooperative in agreement with previous interpretations. It is demonstrated that cooperative units can be evaluated from the relaxation amplitudes. These are of the same order of magnitude as those obtained from static experiments. On the grounds of the present kinetic investigation we can state that the application of the linear Ising model to two-dimensional processes as attempted for the static lipid phase transition is inadequate.

Introduction

The current interest in the physical chemistry of lipid phase transitions appears to be focussed mainly on the equilibrium and the change of thermodynamic properties between the two phases. The kinetic aspects have found relatively little consideration which may partly be due to the apparent complexity of the problem. Nonetheless, kinetic studies of model systems such as liposomes and vesicles may provide pertinent information for the dynamics of

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lipids in their natural membrane environment and their function both as a fluidity switch and a passive transport barrier.

The complexity of the transition kinetics in such systems is reflected by the spectrum of effects as observed with relaxation methods and by the generally tentative approaches to the interpretation of those phenomena. Two groups of applied relaxation techniques and with them the corresponding relaxation rates are to be differentiated: ultrasonic and dielectric relaxation on the one hand [1—5] and temperature-jump relaxation [6—10] on the other. These relaxation effects evidently have something to do with the properties of the bilayer phase transition, but the interpretation on a molecular basis still lacks clarity. Especially, it is necessary to gather excessive evidence for unspecific detection means such as turbidity or sound absorption.

Here we concentrate on the pressure-jump method with observation of turbidity. It is the first detailed application of this method to lipid phase transitions. Before, only preliminary pressure-jump experiments have been reported as a conference abstract [11]. In the following, we present two different sets of results: one on liposomes and one on vesicles. Apparently, due to differences in the aggregate structure, these two preparations show markedly different kinetic characteristics. The results overcome three essential disadvantages of temperature-jump experiments: 1. A dielectric breakdown of the lipid bilayer due to a transient electric field can be excluded [10,12,13]. 2. Pressure as an isotropic quantity has no separate orientation effect on the lipid head groups as an electric field does [4]. 3. Pressure also provides isotropic conditions in the sense that it excludes temporarily different temperatures between the inside and outside of vesicles or liposomes [14] as they may occur in a Joule heating temperature-jump.

Experimental

Lipids

The lipids L-β,γ-dimyristoyl-α-phosphatidylcholine and L-β,γ-dipalmitoyl-α-phosphatidylcholine were purchased from Fluka, Switzerland. In thin-layer chromatography no impurity was detected; therefore the substances were used without further purification.

Preparation of lipid aggregates

For the preparation of multilamellar liposomes, 10 mg dimyristoyl-phosphatidylcholine or dipalmitoyl-phosphatidylcholine were sonicated for 1—2 min at low power in 10 ml bidistilled water at a temperature above the corresponding phase transition temperature. For each experiment a freshly prepared dispersion was used because the suspensions tend to settle during longer times of standing.

Vesicles were prepared by injecting an ethanolic lipid solution into the buffer at a temperature above the phase transition temperature (35 and 50° C) and subsequent dialysis against pure buffer for at least 8 h [15]. Buffer conditions were 0.01 M Tris-HCl (pH 7.5), 0.1 M NaCl and 1 mM NaN_3. The solution remains stable for several days if kept at similar temperatures as during preparation. This was tested by measuring turbidity. In the case of vesicles

the aggregate stability towards pressure-jumps is a critical experimental point, because breaking of the vesicle membrane into fragments might result in reaggregation to large, potentially even multilayered structures. The stability was checked by measuring the mean vesicle radii before and after several pressure-jumps of 70 bar by means of the laser light scattering autocorrelation technique. The mean vesicle radius proved unaffected by the pressure perturbation. But from the dependence of the correlation time on the sampling time it can be estimated that a small proportion of vesicles is broken up into fragments which reaggregate to some unspecified species. Thus each pressure-jump is accompanied by a minute increase in solution turbidity. Undoubtedly this affects the accuracy of the kinetic amplitudes for which reason we chose to normalize these as described below. Since the steepness of transition curves is a sensitive measure for the vesicle size [16] and the integrated amplitudes agree with the thermodynamically obtained transition curves quite well (see Results section) we trust that the bulk of all vesicles retains its shape after the perturbation.

Thermotropic transition curves

Thermotropic transition curves were observed by measuring turbidity with a Cary 1601 photometer or 90° light-scattering intensity with a Farand MK1 fluorometer at 365 nm wavelength. The temperature was scanned at 12 $K \cdot h^{-1}$ from high to low temperature.

Pressure-jump technique

The pressure-jump method with optical detection (turbidity in our case) has been described previously [17]. In this method, static pressure is applied to the sample cell for time intervals long enough to allow chemical equilibration at the elevated pressure level. The pressure-jump back to ambient conditions is accomplished by bursting a metal membrane which seals the autoclave. Bursting pressure was chosen as 150 bar for the liposome solutions and 55 bar for the vesicle solutions. Reproducibility of the bursting pressure is ±10% which is a main source of error in the amplitude determination. Dead-time of the instrument is given by the brass membrane bursting-time of a maximum of 100 μs. For vesicle suspensions the temperature was kept constant (±0.1 K) during each experiment and lowered stepwise to the next constant value.

In the case of multilamellar liposomes a different experimental approach had to be used for the determination of the temperature dependence of decay time constants and pressure-jump amplitudes because of the narrow temperature range of the phase transition. Small pressure-jumps at constant temperature gave only poor reproducibility due to temperature fluctuations which lead to large changes in the observed time constants. We therefore used a pressure jump of approx. 150 bar, which gives a better signal-to-noise ratio and performed the experiments by decreasing the temperature in the cell at a rate of approx. 3 $K \cdot h^{-1}$. Starting above the phase transition temperature we made a pressure-jump every 0.15 to 0.2 K. From the turbidity signal, time constants and amplitudes were determined by a semi-logarithmic linear least-square fit as a first approximation and a subsequent exponential least-square fit for the improvement of these data. For all magnitudes of perturbation we obtained good fits.

On the application of relaxation methods to cooperative structural transitions

In most experiments on structural transitions the degree of transition θ, is an easily determinable quantity. In the case of a simple isomeric transition A $\rightleftharpoons$ B, θ is the following function of the equilibrium constant s:

$$\theta = \frac{s}{1+s} \tag{1}$$

In complex systems like molecular aggregates composed of a large number of elements with a nearest-neighbour interaction (cooperativity), intermediate states between 'all elements in A' and 'all elements in B' occur with a certain probability. In such systems, θ generally depends also on other parameters: $\theta = f(s, \ldots)$. The equilibrium constant, s, is then interpreted as a quantity describing the elementary process of propagation of structural change, and the other parameters are, for instance, nucleation parameters and the number of elements. Since of all processes, propagation steps predominate with by far the highest probability of occurrence, the shift of θ by means of temperature or pressure is, therefore, controlled practically by the equilibrium constant, s, alone. The dependence of θ on s, however, may be complex. As an example, the application of the one-dimensional Ising model to helix-coil transitions can be mentioned [18,19].

For our kinetic experiments we need to know the amplitude $\delta\theta$ as a function of s. At $P_0 = 1$ atm, s is

$$s = \exp\left\{-\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R}\right\} = s_0(T) \tag{2a}$$

and at $P \neq 1$ atm

$$s = s_0(T) \cdot \exp\left\{-\frac{\Delta V^0}{RT}(P - P_0)\right\} \tag{2b}$$

For cooperative structural transitions, ΔH^0 is equal to the calorimetrically determined molar transition enthalpy, ΔH_{cal}, and ΔV^0 is equal to the molar transition volume as obtained from compressibility or density data. The amplitude $\delta\theta$ can be taken from the following equations

$$\left(\frac{\partial\theta}{\partial T}\right)_P = \Gamma\left(\frac{\partial \ln s}{\partial T}\right)_P = \Gamma \cdot \frac{\Delta H_{cal}}{RT^2} \tag{3a}$$

or

$$\left(\frac{\partial\theta}{\partial P}\right)_T = \Gamma\left(\frac{\partial \ln s}{\partial P}\right)_T = -\Gamma \cdot \frac{\Delta V^0}{RT} \tag{3b}$$

For simplicity we set $\delta\theta/\delta \ln s = \Gamma$. In the case of a rapid pressure change, Eqn. 3b needs an amendment for the adiabatic temperature effect. The combination of Eqns. 3a and 3b yields

$$\left(\frac{\partial\theta}{\partial P}\right)_T = -\left(\frac{\partial\theta}{\partial T}\right)_P \cdot \frac{T \cdot \Delta V^0}{\Delta H_{cal}} \tag{4}$$

This relation demonstrates the equivalence of temperature and pressure per-

turbation and represents a convenient tool for kinetic experiments on cooperative transitions. Without the knowledge of the pressure induced transition curve (special equipment is necessary for its determination), Eqn. 4 permits predictions about the amplitude of pressure-jump experiments from the thermotropic transition alone. For structural transitions Eqn. 4 also comprises an interesting parallel with the Clausius-Clapeyron relation for the coexistence curve between two phases, 1 and 2, in a phase diagram:

$$\frac{T \cdot \Delta V^0}{\Delta H_{cal}} = \left(\frac{dT}{dP}\right)_\theta \tag{5}$$

Obviously the slopes of transition curves reach maxima at the midpoint of transition. These maxima we define as $[\partial\theta/\partial T)_P]_m$ and $[(\partial\theta/\partial P)_T]_m$ and the corresponding amplitude factor as Γ_m. This midpoint amplitude factor, Γ_m, can serve as a measure for the cooperativity of the investigated system. If we assume artificially a two-state mechanism $A \rightleftharpoons B$ (which is never true for complex cooperative transitions) the amplitude factor is given by $\Gamma = \theta(1-\theta)$ which is 0.25 for $\theta = 0.5$. Thus, Eqns. 3a and 3b will read at the midpoint of transition

$$\left(\frac{\partial\theta}{\partial T}\right)_{P,T_m} = \frac{\Delta H_{vH}}{4RT_m^2} \tag{6a}$$

and

$$\left(\frac{\partial\theta}{\partial P}\right)_{T,P_m} = -\frac{\Delta V_{vH}}{4RT} \tag{6b}$$

The van't Hoff quantities ΔH_{vH} and ΔV_{vH} are defined by these equations. The real meachnism may imply $\Gamma \neq \theta(1-\theta)$. However, according to essentially all plausible models, Γ will assume a maximum Γ_m at $\theta = 1/2$, i.e. T_m or P_m, and we find from the comparison between an artificial two-state model (Eqns. 6a and 6b) and an unspecified real mechanism (Eqns. 3a and 3b at midpoint conditions)

$$4\Gamma_m = \frac{\Delta H_{vH}}{\Delta H_{cal}} = \frac{\Delta V_{vH}}{\Delta V^0} \tag{7}$$

The ratio $\Delta H_{vH}/\Delta H_{cal}$ has frequently been used as a measure for cooperativity [8,16]. Its close relation to the amplitude factor Γ_m allows a decision about the extent of the observed cooperativity. Evidently $4\Gamma_m = 1$ for a non-cooperative process.

Results

We report results on vesicle as well as liposome solutions. They have to be treated differently because transition curves of vesicles are relatively smooth (Figs. 1c, 2c) whereas those of liposomes are very steep (Figs. 3b, 4b). As a consequence, kinetic data for vesicles can be treated as outlined above; on the other hand, those for liposomes demand a special evaluation as described below.

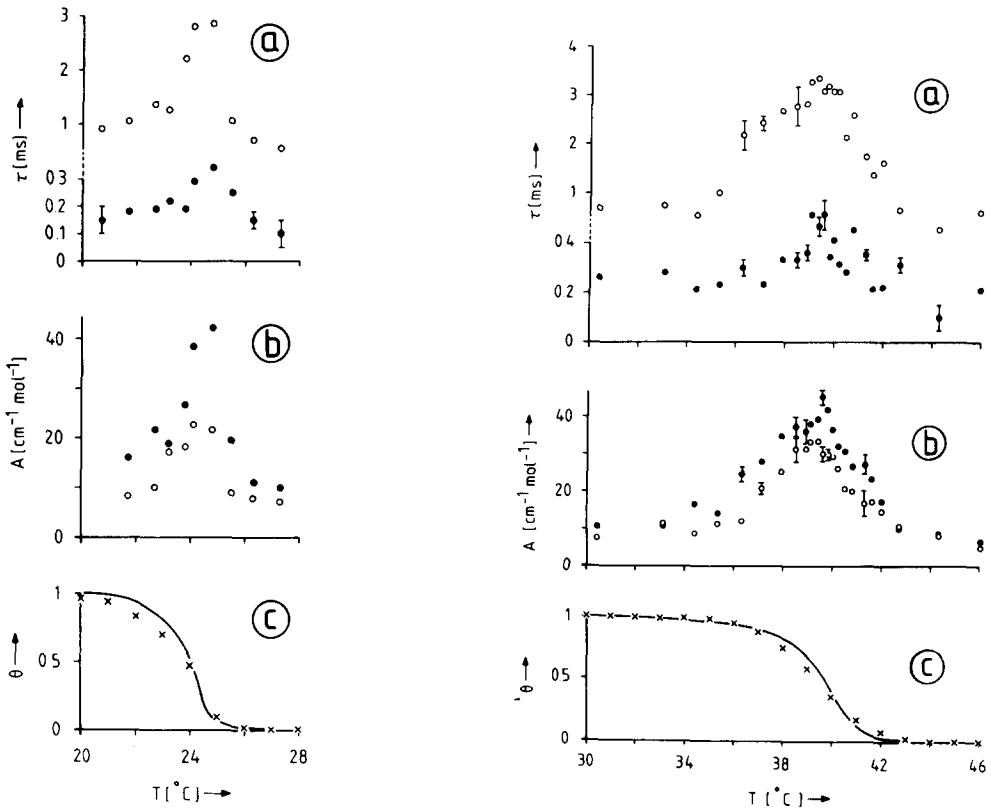

Fig. 1. Pressure-jump experiments on dimyristoyl-phosphatidylcholine (DMPC) vesicles of 28 nm radius. Lipid concentration was 1.8 mM in 0.1 M NaCl/0.01 M Tris · HCl (pH 7.5)/1 mM NaN_3. (a) Relaxation times as a function of temperature, ○, slow effect; ●, fast effect. (b) Relaxation amplitudes from turbidity change at 365 nm after pressure-jumps of 55 bar. The symbols correspond to those of (a). (c) Normalized transition curve (solid line) as obtained from light scattering at 365 nm. Crosses represent numerical integration of the amplitudes of (b) according to Eqn. 9.

Fig; 2. Pressure-jump experiments on dipalmitoyl-phosphatidylcholine (DPPC) vesicles of 25 nm radius. Lipid concentration was 2 mM in 0.1 M NaCl/0.01 M Tris · HCl (pH 7.5)/1 mM NaN_3. a—c as in Fig. 1, symbols as in Fig. 1.

Vesicles

Thermotropic transition curves of the present vesicle preparations were obtained from scattered-light intensity as a function of temperature. The experimental transition curves were normalized by attributing the plateau at $T > T_m$ to $\theta = 0$ (fluid state) and the corresponding plateau at $T < T_m$ to $\theta = 1$ (solid state), see Ref. 16. These normalized curves are plotted in Figs. 1c and 2c.

For comparison, our kinetic results on dimyristoyl-phosphatidylcholine and dipalmitoyl-phosphatidylcholine vesicles are depicted above the static transition curves on the same temperature scale in Figs. 1a,b and 2a,b. For all solutions we observed two distinct relaxation effects. However, both of these phenomena are clearly associated with the phase transition as the peaked τ vs. T dependence demonstrates. To exclude the possibility of aggregation

from these two processes, we tested the concentration dependence of τ by diluting the same preparation and thereby leaving the aggregate dimensions unchanged. Neither of the two relaxations exhibited different time constants. Further evidence for the close relation between the observed processes and the phase transition can be drawn from the good agreement between the thermotropic transition curve and the integrated pressure-jump amplitudes. This integration is achieved by means of Eqn. 4. The amendment for adiabatic pressure release would be

$$\left(\frac{\partial\theta}{\partial P}\right)_S = -\left(\frac{\partial\theta}{\partial T}\right)_P \cdot T\left\{\frac{\Delta V^0}{\Delta H_{cal}} - \frac{\alpha_p}{\rho c_p}\right\} \tag{8}$$

where α_p is the thermal expansion coefficient, ρ the density and c_p the specific heat capacity. A rough estimation with data from Ref. 20 yields $\Delta V/\Delta H_{cal} \approx 6.8 \cdot 10^{-10}\ m^2 \cdot N^{-1}$ and for dilute aqueous solutions we find $\alpha_p/\rho c_p \approx 6.2 \cdot 10^{-11}\ m^2 \cdot N^{-1}$. This deviation of 10% falls within the experimental error of the amplitude determination and is therefore disregarded. Integration of the simplified Eqn. 8 yields then

$$\theta = \int_{T(\theta=0)}^{T(\theta)} \left(\frac{\partial\theta}{\partial T}\right)_P dT = -\frac{\Delta H_{cal}}{\Delta V} \cdot \int_{T(\theta=0)}^{T(\theta)} \frac{1}{T}\left(\frac{\partial\theta}{\partial P}\right)_T dT \tag{9}$$

From the data of Ref. 21 we can deduce a linear relationship between θ and turbidity, A, as a first approximation:

$$\theta = \frac{A - A_f}{A_s - A_f} \tag{10}$$

(subscripts s for solid, f for fluid phase). The change in turbidity is

$$\delta A \approx (A_s - A_f)\,\delta\theta + \delta A_f \tag{11}$$

if we assume that $\theta(\delta A_s - \delta A_f)$ is much smaller than the other terms in the transition midpoint vicinity. This is justified since the limiting slopes of turbidity vs. temperature are very similar for $T < T_m$ and $T > T_m$ so that $\delta A_s \approx \delta A_f$. Under these conditions we can substitute

$$\delta\theta = \frac{\delta A - \delta A_f}{A_s - A_f} \tag{12}$$

into Eqn. 9 which enables us to integrate numerically. The integrated sums of the kinetic amplitudes ($A_s + A_f$) are plotted as crosses in Fig. 1c, 2c for comparison with the static transition curve. There is good agreement for such a relatively crude approach (δP is larger than appropriate for the above approximations).

From the experimental observation it was confirmed that the sum of both relaxation amplitudes is identical with the entire shift of the equilibrium as indicated by turbidity. No further relaxation effect was found to be buried in the dead-time of the instrument.

For reasons which are explained in the experimental section, the amplitude determination remains relatively uncertain. However, according to Eqn. 7 we can offer an order of magnitude for the cooperative unit. We find it to be

between 70 and 150 for the dimyristoyl-phosphatidylcholine vesicles, which is to be compared with 90 to 140 from a recent report [16].

Liposomes

In all kinetic experiments on liposome dispersions we have applied large perturbations of the equilibrium. Nonetheless, we report about the outcoming effects as 'relaxations'. Fig. 3a shows the total relaxation amplitude of dimyristoyl-phosphatidylcholine liposomal dispersions as a function of temperature. The increase in the amplitude between 23.5 and 25° C is due to the phase transition at a pressure of 1 bar, whereas the decrease between 26.5 and 28° C indicates the transition at 150 bar. This is seen from a comparison between Figs. 3a and 3b following the depicted arrows. A pressure increase of 150 bar shifts the transition temperature of dimyristoyl-phosphatidylcholine by approx. 3.25 K, which gives 46.2 bar · K^{-1} for dP_m/dT_m. This is in good agree-

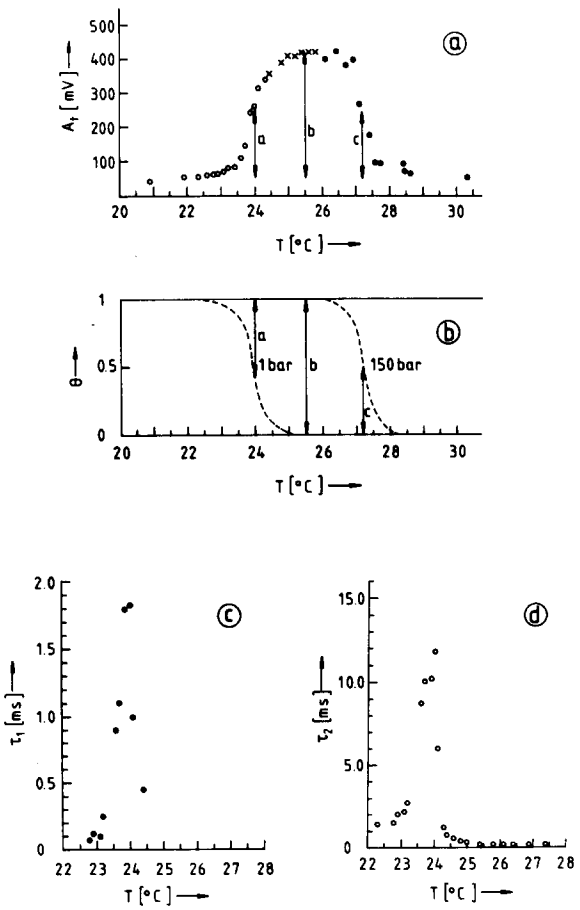

Fig. 3. DMPC liposomes. (a) Total relaxation amplitude as a function of temperature ($\delta P \approx 150$ bar). (b) Normalized transition curves from the total relaxation amplitudes. The arrows in a and b stand for jumps from solid state (150 bar) to $\theta = 0.5$ (1 bar), solid state (150 bar) to fluid state (1 bar) and $\theta = 0.5$ (150 bar) to fluid state (1 bar). (c) Fast relaxation time τ_1 as a function of temperature. (d) Slow relaxation time τ_2 as a function of temperature. Symbols as in Fig. 1.

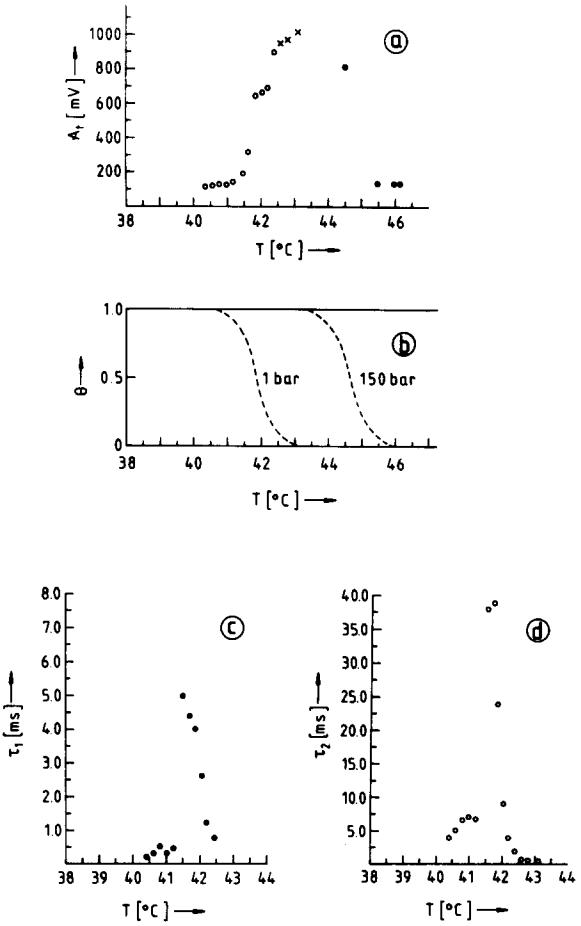

Fig. 4. DPPC liposomes. a—d see legend to Fig. 3.

ment with the value of 49 bar · K^{-1} reported by de Smedt et al. [20].

Two relaxation times could be evaluated in the temperature range of the transition. Both have a maximum at the midpoint of the transition (see Figs. 3c and 3d). Both relaxation times for dimyristoyl-phosphatidylcholine liposomes are longer than those for the monobilayer vesicles. In the temperature range below 24° C (circles in Fig. 3a), the total measured relaxation amplitude is always larger than the sum of the two amplitudes calculated from the relaxation curves. Additional relaxation processes with time constants shorter than the pressure-drop time therefore have to be assumed. Between 24.3 and 25.8° C (crosses in Fig. 3a), the relaxation curve can be described by one exponential the time constant of which decreases gradually from approx. 700 to approx. 100 μs. Possibly, both relaxation times become similar in this temperature range, due to different activation energies. The smaller τ values at the high-temperature end of the transition, as compared to the low-temperature side, may arise from the application of a large perturbation. This might represent an analogy to linear systems, for which a dependence of the relaxation rate on the perturbation size has been described previously [22]. Above 26° C (filled

circles in Fig. 3a) the relaxation amplitude decreases again. Only one relaxation time can be evaluated (approx. 100 μs), but the total relaxation amplitude is larger than the calculated one, so that again, additional relaxation processes are present. With dipalmitoyl-phosphatidylcholine liposomes similar results were obtained (see Figs. 4a—d). For dP_m/dT_m a value of 54 bar $\cdot$ K^{-1} was calculated from the transition curves in Fig. 4b. This is somewhat larger than the value reported by Srinivasan et al. (44 bar $\cdot$ K^{-1}). The relaxation times for dimyristoyl-phosphatidylcholine are larger than those observed for dipalmitoyl-phosphatidylcholine liposomes in accordance with the findings for vesicle suspensions. Between 41.5 and 41.9°C, i.e around the midpoint of the transition for dimyristoyl-phosphatidylcholine, a third relaxation time of approx. 100 μs could be observed, which becomes shorter than the instrumental dead-time below and above this temperature range.

Discussion

We can summarize our results as follows. We resolve two (or three, respectively) cooperative relaxation processes in the phase transition region. Cooperativity of these processes is indicated by the peaked dependence of the relaxation times on the temperature as well as by the amplitude maximum in the transition midpoint which is higher than that for a noncooperative process. The amplitude behaviour is readily seen from the static transition curves. For the behaviour of relaxation times of transitions in two-dimensional systems, two major model-bound interpretations have been offered [23,24], and a critical elongation of time constants in lipid bilayer phase transitions has been discussed recently by Mitaku et al. [25].

Empirically, we find that for phase transitions in lipid vesicles, τ is a linear function of $\sqrt{\theta(1-\theta)}$ (Fig. 5). This implies a symmetrical shape of τ vs. θ. For linear systems, such a symmetrical τ vs. θ dependence has been investigated and interpreted [19,26]. Yet, in the one-dimensional case (i.e. helix-coil transitions), τ is linear with $\theta(1-\theta)$. This clearly shows that the application of the

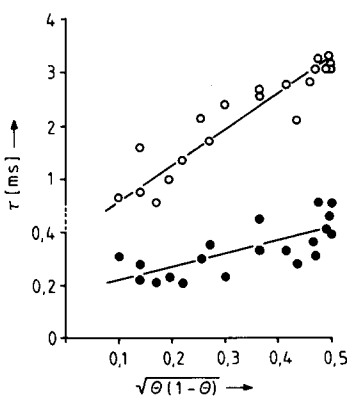

Fig. 5. Relaxation times for the DPPC vesicle solution of Fig. 2 as a function of $\sqrt{\theta(1-\theta)}$. ○, slow effect; ●, fast effect.

one-dimensional Ising model to two-dimensional problems as attempted by Marsh et al. [27] is inadequate.

The larger τ values for liposome solutions may be explained by the sharper transition as compared to vesicles, again in analogy to linear systems [19].

As to the difference in relaxation times between the two lipid systems, we found that for both vesicle and liposome solutions, all corresponding τ values were larger for dipalmitoyl-phosphatidylcholine than for dimyristoyl-phosphatidylcholine. In the temperature range of the transition of dipalmitoyl-phosphatidylcholine liposomes, a third relaxation process connected with the transition could be observed. As judged from the difference between the total amplitude and the sum of amplitudes for the resolved effects, this process is also present, but unresolvable in dimyristoyl-phosphatidylcholine liposomes.

Those processes outside of the transition temperature range are very fast and have very small amplitudes. Evidently, they do not belong to the transition itself. They have very little temperature dependence and probably also occur in the transition range where they cannot be detected due to their small contribution to the amplitude. Their molecular origin remains unclear.

A number of recent reports have presented evidence that the interpretation of kinetic behaviour associated with the lipid phase transition can be subdivided into two categories: the nanosecond effects and the micro- and millisecond effects. Due to experimental restrictions, the former ones have not been well characterized. The time-scale coincidence of those phenomena with isomerization effects in aliphatic chains leads all authors to the conclusion that intrachain rotations (kink formations) are responsible for ultrasonic absorption in the MHz region [2,3,5]. Unfortunately, these effects are buried in a spectrum of ultrasonic absorptions and are certainly overlapping with head-group motion [1,2,4].

Clear evidence for the relaxation time and amplitude dependence on the degree of transition of lipid bilayers has been demonstrated in two publications only [8,10]. In both cases, the Joule-heating temperature-jump technique was applied to dimyristoyl-phosphatidylcholine and dipalmitoyl-phosphatidylcholine liposomes. Tsong and Kanehisa [10] reported three major concentration-independent processess: in the μs-, the 10 ms- and the 1 s range. The latter two show the mentioned τ-θ profile. The μs effect we cannot resolve due to a 100 μs dead-time. Contrary to the observations of Tsong and Kanehisa, our cooperative effects lie in the 0.1—1 ms and the 3—40 ms ranges. We have shown the equivalence of pressure and temperature perturbation on the equilibrium (see Eqn. 4) and therefore have to assign deviations to experimental differences. As discussed in the introduction, the pressure-jump eliminates the possibility of dielectric breakdown and acts completely isotropically, in contrast to the Joule temperature-jump. This may eliminate artifacts. As to the 1 s range effects: indeed, we observed such cooperative relaxations on liposome solutions when we used a buffer with 0.1 M KCl or NaCl instead of very low ionic strength given by the buffer itself. These effects never occurred in vesicle solutions. We conclude that this slow relaxation may be due to a change in the state of aggregation of the liposomes.

A molecular model including all effects reported so far cannot be offered at the present stage. Up to now, it has not been shown whether the effects

observed in the ultrasound time range are cooperative. Also, attempts to correlate water permeation and effects in the ms range have to be considered with reserve because they have been made for measurements outside the phase transition region [12,14,28,29].

However, we feel that our results and their comparison with results of other authors indicate that the pressure-jump method has some clear advantages in comparison with other relaxation methods. Therefore, it will be a useful tool for the kinetic study of lipid phase transitions.

Acknowledgements

The authors greatly appreciate stimulating discussions with Professor Th. Ackermann, Professor G. Schwarz and Dr. S. Stankowski. The technical assistance of Mrs. C. Hildebrand is also gratefully acknowledged. A.B. was supported by the Deutsche Forschungsgemeinschaft.

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