

Relaxation Kinetics of Lipid Membranes and Its Relation to the Heat Capacity

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ABSTRACT We investigated the relaxation behavior of lipid membranes close to the chain-melting transition using pressure jump calorimetry with a temperature accuracy of $\sim 10^{-3}$ K. We found relaxation times in the range from seconds up to about a minute, depending on vesicular state. The relaxation times are within error proportional to the heat capacity. We provide a statistical thermodynamics theory that rationalizes the close relation between heat capacity and relaxation times. It is based on our recent finding that enthalpy and volume changes close to the melting transition are proportional functions.

INTRODUCTION

Most lipids that are found in cell membranes have chain-melting temperatures close to a temperature regime of biological relevance. When going through the phase transition, several observables of the system change. The enthalpy increases by ~ 20 – 40 kJ/mol, the specific volume increases by 4%, and the area by $\sim 25\%$ (Heimburg, 1998). At the melting temperature, the heat capacity reaches a maximum. It is known that several system properties of membranes change in a way that is closely related to the heat capacity, namely other response functions as the isothermal volume and area compressibilities, the bending elasticity, but also the relaxation times which are maximum at the melting point. The lipid membrane forms the matrix in which proteins of various function and activity are imbedded. Certain enzymes possess functions that respond to melting processes, including phospholipase A_2 (Burack et al., 1993). Protein activity is often related to changes in cross section or volume. Therefore, it is likely that they respond to changes in compressibility in their direct environment with a time characteristic similar to relaxation processes of the lipid matrix. For this reason, it seems interesting to understand time scales of state changes of lipid membranes.

Since the classical paper of Tsong and Kanehisa (1977), relaxation times of lipid membranes close to the chain-melting transitions have been investigated by various authors (Elamrani and Blume, 1983; Blume and Hillmann, 1986; van Osdol et al., 1989, 1991a). Most data available in the literature are based on perturbations induced by pressure or temperature changes being monitored by optical means such as light scattering, fluorescence, or infrared spectral changes (Tsong and Kanehisa, 1977; Elamrani and Blume, 1983; Blume and Hillmann, 1986). These methods are able to record on a fast time scale. It is, however, not easy to obtain a good control over

absolute temperature in an optical setup. Lipid transitions may be very cooperative, with transition half width of 0.05 K for multilamellar vesicles up to ~ 1 K for unilamellar vesicles. Therefore, it is difficult to obtain quantitative information at the transition peak. Because optical parameters are only indirect indicators for the state of the system, it is not always clear which property of the system is observed. For this reason, periodic volume perturbations have been used to monitor the response of the system in a calorimeter (Johnson et al., 1983; van Osdol et al., 1989, 1991a). This method has the advantage of being extremely precise in absolute temperature. However, due to the periodic perturbation, the pressure is not constant, and the state of the system is not well defined.

Here, we present a pressure perturbation method to monitor relaxation times in a calorimeter. These measurements have the advantage of always having well-defined temperature and pressure with an accuracy of 0.001 K and 0.1 bar. These uncertainties are much smaller than the transition half width of unilamellar vesicles. Thus, we are able to obtain very good numbers for relaxation times close to the heat capacity maximum. The quality of the data allows for the comparison with a linear nonequilibrium thermodynamics model that relates the heat capacity very closely to the relaxation times.

Our paper is structured as follows. First, we provide an extended theory part with a new approach to understand relaxation processes of cooperative lipid melting events. The major outcome of this section is that the relaxation times of lipid systems close to the chain melting transition are proportional to the excess heat capacity and can therefore be estimated from calorimetric profiles. For readers with rather experimental interests who are willing to accept the outcome of the theory part, it is possible to directly jump to the second section of this paper. This experimental part presents a new calorimetric pressure-jump technique that relates calorimetric heat capacities to the relaxation times and confirms the theoretical predictions.

MATERIALS AND METHODS

Lipids were purchased from Avanti Polar Lipids (Birmingham, AL) and were used without further purification. Lipids were measured in a 10-mM HEPES buffer with 1 mM EDTA at pH 7.0. Lipid concentrations were up

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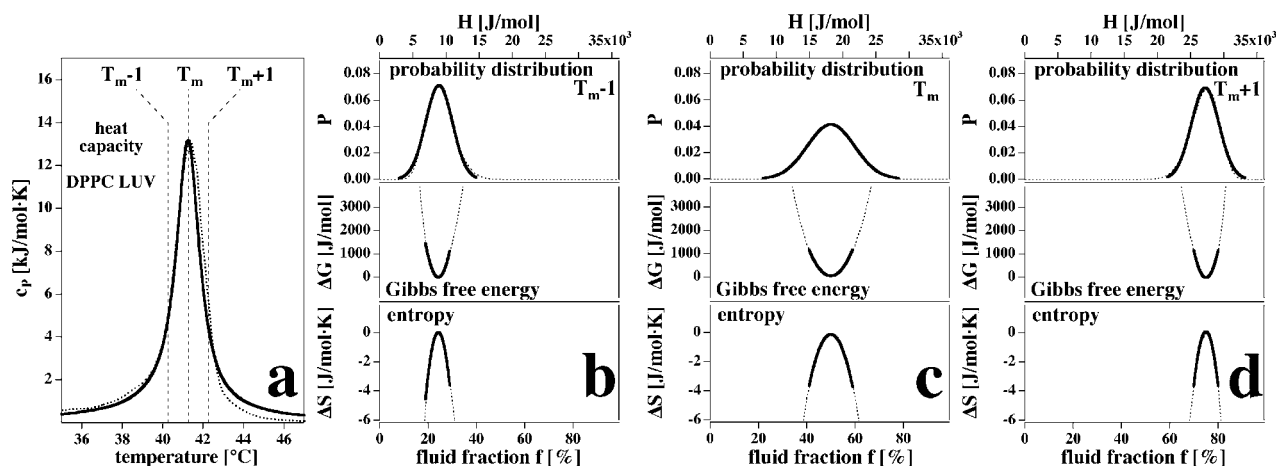


FIGURE 1 (a) Experimental heat-capacity profile of DPPC large unilamellar vesicles (*dotted line*) and a fit from a two-state model (*solid line*) (Ivanova and Heimburg, 2001). (b) Probability distribution of enthalpy states around the average value, taken from a Monte Carlo simulation of large unilamellar vesicles of DPPC one degree below the melting temperature at $T_m - 1^\circ$ (Ivanova and Heimburg, 2001). The thick line represents a Gaussian fit to the distribution of states. Center: Gibbs' free energy profile around the equilibrium value. The thick line represents a fit with a quadratic function. Bottom: Entropy around the equilibrium value. The thick line represents a fit with a quadratic function. (c) Same as b at the melting temperature T_m . (d) Same as b one degree above the melting temperature at $T_m + 1^\circ$.

to 100 mM. Large unilamellar vesicles (LUV) were produced using an AVESTIN extruder. To increase the LUV concentration, the lipid dispersion was centrifuged in a vacuum ultracentrifuge. Heat capacities were recorded on a VP-calorimeter from Microcal, Inc. (Northampton, MA) at scan rates of 5 K/hr and 0.2 K/hr for the very cooperative transitions of multilamellar vesicles. The time constant of a VP-calorimeter is ~ 5 s. Pressure calorimetry was performed on this instrument using a self-built pressure capillary. This cell has already been used by Ebel et al. (2001). The pressure was controlled with nitrogen gas and measured with a sensor (EBM 6045) from Nova Swiss (Effretikon, Switzerland). During DSC scans, the pressure was maintained with an error of less than 0.5%, which displayed a slight systematic temperature dependence. Because the transition half-width of a single lipid usually was smaller than 1 K, this error was negligible for these systems. Relative temperature changes of c_p maxima, induced by pressure, can be determined with a precision of ~ 0.001 K.

Pressure jumps were performed in the isothermal mode of the calorimeter. After a pressure jump, the new pressure was achieved within less than 0.1 s (much faster than the time resolution of our experiment, which is larger than 1 s).

Evaluation of relaxation data requires that the temperature during the time of the calorimetric response is constant. This has been checked for each experiment. Usually this boundary condition is fulfilled when pressure jumps are obtained with negative change of pressure. Under these conditions, the lipid response leads to chain melting, resulting in a minute decrease in temperature, which is immediately compensated by the calorimeter. Heat increases cannot be compensated at a similar rate. Therefore, only few of our data have been obtained with positive pressure jumps. Furthermore, if the sample absorbs too much heat, the maximum power of the feedback mechanism might be exceeded. This also leads to changes in absolute temperature. These data have not been analyzed. Usually the temperature stayed constant within ~ 0.002 K (see Fig. 3). Therefore, we assumed that the pressure jump did not significantly affect the cell temperature and that the physical state of the lipid was well defined.

Monte-Carlo simulations were performed on the basis of a two-state Ising model (Doniach, 1978; Sugar et al., 1994; Heimburg and Biltonen, 1996; Ivanova and Heimburg, 2001) using a 31×31 triangular lattice with periodic boundary conditions. Monte Carlo steps for switching states were computed using a standard Metropolis algorithm. During the Monte-Carlo simulation, the system variables (enthalpy, number of fluid molecules, . . .)

fluctuate around the equilibrium value. From the fluctuations, one can calculate the probability distribution of states with given enthalpy, $P(H)$, and the heat capacity (cf. Fig. 1). Details of this procedure were described in detail by Ivanova and Heimburg (2001).

THEORY

A well-known statistical thermodynamics theorem (fluctuation-dissipation) relates the heat capacity to the fluctuations in enthalpy (Hill, 1960):

$$c_p = \frac{\overline{H^2} - \overline{H}^2}{RT^2}, \quad (1)$$

where \overline{H} is the mean enthalpy at given pressure p and temperature T . If the lipid system is large enough and is not at a critical point, one can approximate the distribution of states around the equilibrium value by a Gaussian distribution,

$$P(H - \overline{H}) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left(-\frac{(H - \overline{H})^2}{2\sigma^2}\right), \quad (2)$$

where σ is the width of the distribution, which, for a Gaussian distribution, is identical to $\sigma^2 = (\overline{H^2} - \overline{H}^2)$. In Fig. 1 a, an experimentally obtained heat-capacity profile of large unilamellar dipalmitoylphosphatidylcholine (DPPC) vesicles is given and is compared to a theoretical calculation from a lattice two-state Ising model (details are given in Ivanova and Heimburg (2001); see also Materials and Methods). For the calculations, a triangular lipid matrix was generated in a computer. Lipids may either assume a gel or a fluid state. No intermediate states are considered. The equilibrium states of the membrane are then calculated by

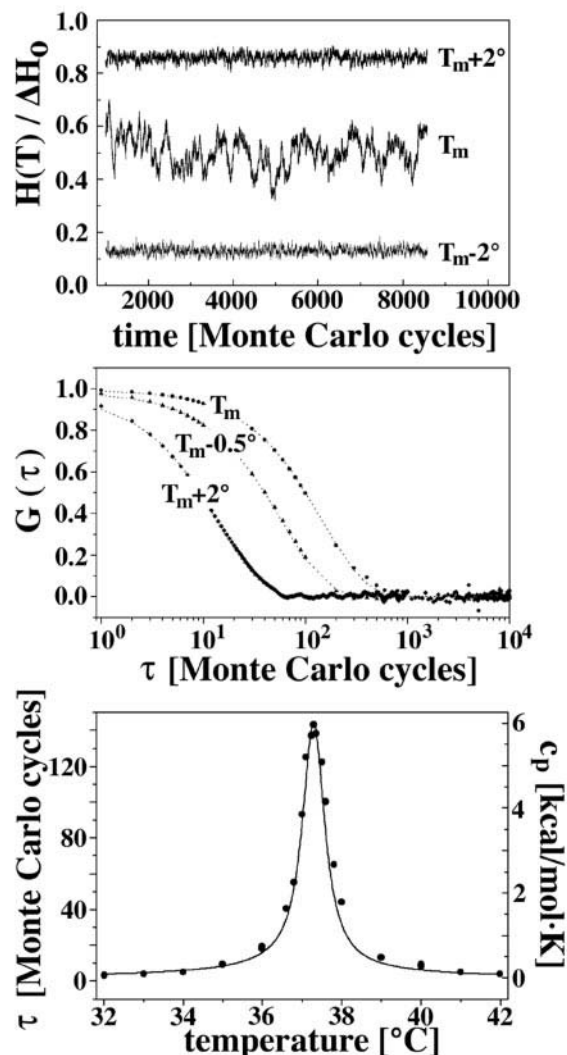


FIGURE 2 Top: Enthalpy fluctuation of unilamellar vesicles at the melting point, T_m , at $T_m - 2^\circ$, and at $T_m + 2^\circ$, deduced from Monte Carlo simulations (Ivanova and Heimburg, 2001) (cf. Fig. 1). Fluctuation traces of this kind were used to calculate the distribution of enthalpy states in Fig. 1. Fluctuations at T_m have maximum amplitude, with a slow time scale. At the two other temperatures, the amplitude of the noise is small and the time scale is fast. Center: The time scale of the noise can be obtained from autocorrelation analysis. Three autocorrelation functions for Monte Carlo noise at T_m , at $T_m - 0.5^\circ$, and $T_m + 2^\circ$ are shown. Dotted lines represent single exponential fits. At T_m , the relaxation time is maximum. Bottom: Time constant of Monte Carlo noise (from the autocorrelation analysis, in units of Monte Carlo time) and heat capacity, c_p , as a function of temperature. The proportional relation between heat capacity and relaxation times is verified in the computer experiment. ΔH_0 is the calorimetric excess melting enthalpy.

Monte Carlo simulations. Three parameters enter these simulations: the transition enthalpy, ΔH_0 , the melting temperature, T_m , and the transition half width, all of which are experimental numbers. These simulations also yield the fluctuations in enthalpy around the equilibrium state (Fig. 2a). It is obvious that the simple lattice model is able to

describe the chain-melting reaction quite well (Fig. 1a). The distribution of states with different enthalpy, $P(H - \bar{H})$, obtained from these simulations at three different temperatures below, at and above the melting temperature, is described by a Gaussian distribution of states, as noted above (Fig. 1, b–d). Thus, in the following, we will assume that the statement that enthalpy fluctuations are of Gaussian nature is correct.

Therefore, the heat capacity is given by

$$c_p = \frac{\sigma^2}{RT^2}. \quad (3)$$

The Gibbs free energy of a state with mean enthalpy \bar{H} can be derived from the distribution of enthalpy states (Lee and Kosterlitz, 1991) by

$$G(H - \bar{H}) = -\ln P(H - \bar{H}) + \text{const.}, \quad (4)$$

which leads to

$$G(H - \bar{H}) = \frac{(H - \bar{H})^2}{2\sigma^2} + \text{const.}, \quad (5)$$

when the distribution of states is Gaussian. Because the entropy of this distribution is given by $S = (H - G)/T$, we obtain

$$\begin{aligned} S(H - \bar{H}) &= \frac{(H - \bar{H})}{T} - \frac{(H - \bar{H})^2}{2T\sigma^2} - \frac{\text{const.}}{T} \\ &\approx -\frac{(H - \bar{H})^2}{2T\sigma^2} \end{aligned} \quad (6)$$

for small σ (large enough system). Thus, the entropy is a harmonic potential. The quadratic term of the entropy is dominant in the proximity of equilibrium and the linear terms can be neglected. The maximum of the entropy defines the equilibrium state of the system. Fig. 1 (panels b–d) displays the Gibbs free energy and the entropy at three different temperatures obtained from fits of the two-state statistical thermodynamics model (Ivanova and Heimburg, 2001). It is obvious that both functions can be fitted with quadratic functions (fat lines).

In linear nonequilibrium thermodynamics, the thermodynamics forces, X_i , that drive the system back to equilibrium can be derived from the entropy from derivatives with respect to the fluctuations, α_i , of the system, which can generally be written as

$$X_i = \sum_j \left(\frac{\partial^2 S}{\partial \alpha_i \partial \alpha_j} \right)_0 \alpha_j, \quad (7)$$

whereas the fluxes, J_i , i.e., the time-dependent changes of the fluctuations are given by

$$J_i = \frac{d\alpha_i}{dt} = \sum_j L_{ij} X_j, \quad (8)$$

introducing phenomenological coefficients L_{ij} that relate the thermodynamics fluxes to the forces. Because, in lipid systems volume, area and enthalpy changes in the melting transition are proportional functions (Heimburg, 1998; Ebel et al., 2001) there is only one independent fluctuation, $\alpha = (H - \bar{H})$, and the thermodynamic force is given by

$$X(H - \bar{H}) = \left(\frac{\partial^2 S(H - \bar{H})}{\partial (H - \bar{H})^2} \right)_0 (H - \bar{H}) = - \frac{(H - \bar{H})}{T\sigma^2}. \quad (9)$$

The flux of enthalpy back to equilibrium is given by the phenomenological equation

$$\frac{d(H - \bar{H})}{dt} = L \cdot X(H - \bar{H}) = -L \cdot \frac{(H - \bar{H})}{T\sigma^2}, \quad (10)$$

and thus the time dependence of the relaxation is given by the single exponential function

$$\begin{aligned} (H - \bar{H})(t) &= (H - \bar{H})(0) \cdot \exp\left(-\frac{L}{T\sigma^2} t\right) \\ &\equiv (H - \bar{H})(0) \cdot \exp\left(-\frac{t}{\tau}\right), \end{aligned} \quad (11)$$

introducing a relaxation time, τ . Because $\sigma^2 = RT^2 c_p$, it follows for the relaxation time,

$$\tau = \frac{RT^3}{L} c_p \equiv \alpha c_p, \quad (12)$$

and the relaxation time close to the chain-melting transition of lipids becomes a proportional function of the heat capacity with a proportionality constant $\alpha = RT^3/L$.

In the following, we will show that this relation is correct for the fluctuations generated in the computer simulations. As mentioned above, the enthalpy of the computer matrix fluctuates during a Monte Carlo simulation as a function of computer time. The time scale of the fluctuations contains information on the relaxation times. In Fig. 2 (*top*) we show the fluctuations in enthalpy, obtained during a Monte-Carlo simulation (same simulation as performed for Fig. 1), at three different temperatures, T_m , $T_m - 2$, and $T_m + 2$. The time scale given at the x-axes corresponds to the number of Monte Carlo cycles. Obviously, the fluctuation amplitude (related to the heat capacity) and the time scale of the noise (related to the relaxation times) are very different at T_m as compared to temperatures outside the melting regime. The relaxation times of the fluctuations can be analyzed with autocorrelation analysis of the noise, as shown in Fig. 2 (*center*). The autocorrelation function, $G(\tau)$ is given by

$$G(\tau) = \frac{\int_0^\infty (H(t) - \bar{H}) \cdot (H(t + \tau) - \bar{H}) dt}{\int_0^\infty (H(t) - \bar{H})^2 dt}. \quad (13)$$

This function has a value between 1 at $\tau = 0$ and a value of 0 when τ approaches ∞ . The autocorrelation function can be

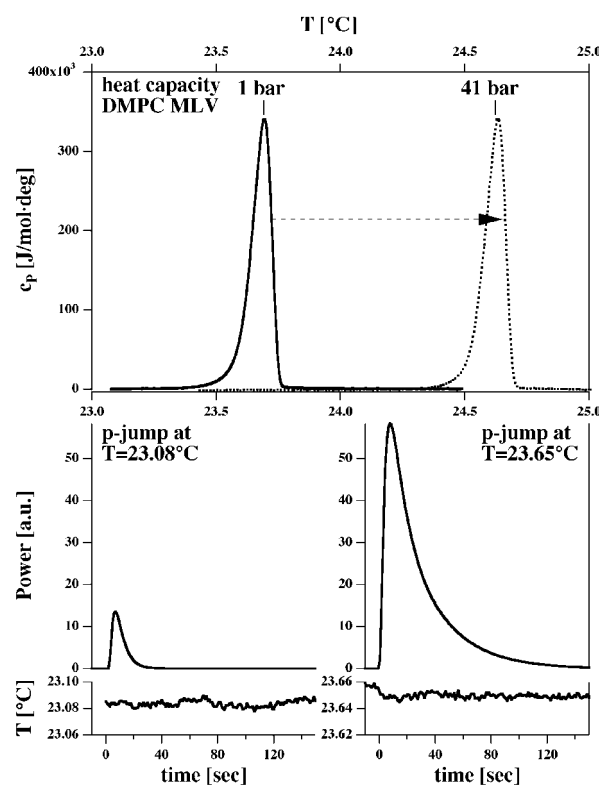


FIGURE 3 *Top*: Calorimetric traces of multilamellar vesicles of DMPC at atmospheric pressure (1 bar) and at 41 bar. The c_p traces shift by about one degree. *Bottom left*: Calorimetric response after a pressure jump from 41 bar to 1 bar for a DMPC MLV dispersion at $T = 23.08^\circ\text{C}$. *Bottom right*: Calorimetric response after a pressure jump from 41 bar to 1 bar for a DMPC MLV dispersion at $T = 23.69^\circ\text{C}$. Note the difference in amplitude and in the time dependence of the relaxation.

fitted with a single exponential function, which yields a value for the relaxation time. It can be seen that the relaxation time is at maximum at T_m . The values for the relaxation times from the autocorrelation analysis are given in Fig. 2 (*bottom*), together with the calculated heat capacity profile (cf. Fig. 1). The proportional relation between heat capacity and relaxation time in a computer experiment therefore can be verified in Monte Carlo simulations.

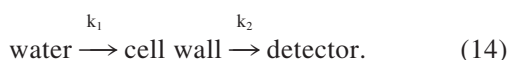
RESULTS

Analyzing pressure jump data

To demonstrate that the proportional relation also holds in experimental systems, we investigated the relaxation behavior of different lipid preparations in a pressure jump calorimeter. The main advantage of this new technique is its very good temperature accuracy in the range of 10^{-3} K throughout the whole relaxation process (seconds to minutes). Because the pressure jump itself is faster than 0.1 s, we can obtain information about processes that are slower.

If we run calorimetric scans for dimyristoylphosphatidylcholine multilamellar vesicles (DMPC MLV) with and

without pressure, a shift of the heat capacity maximum can be observed (Ebel et al., 2001). Applying 40 bar excess pressure shifts the transition by 0.93 K (Fig. 3, *top*). Let us now assume that the experimental temperature is 23.08°C. At this temperature, the samples at both 1 bar and 41 bar are in the gel state. Thus, a pressure jump (41 bar) \rightarrow (1 bar) does not change the state of the lipid, and no lipid response is expected (Fig. 3, *bottom left*). Thus, a signal determined at this temperature mainly originates from the experimental setup, namely from the response of water and the pressure cell walls. We assume that the release of heat from water is very fast (much faster than the time scale of our setup), because the thermal diffusion coefficient of water is high. Thus, heat release from water is instantaneous. We therefore take this experiment as a means to determine the response times of the calorimetric setup. To do so, we assumed the following scheme for the heat released after a pressure jump:



Here, it is assumed that changing the pressure leads to a minor release (or absorption) of heat, which is instantaneous, i.e., the heat is released as a δ pulse. This heat is transferred to the cell wall with a time constant k_1 and then released from the cell wall to the detector with a time constant k_2 . k_1 and k_2 are time constants of the calorimeter. It can easily be shown that this relaxation scheme leads to the following time dependence for the heat release (power)

$$P_{\text{H}_2\text{O}}(t) = P_{\text{H}_2\text{O}}^0 (\exp(-k_1 t) - \exp(-k_2 t)) \quad t \geq 0$$

$$P_{\text{H}_2\text{O}}(t) = 0 \quad t < 0 \quad (15)$$

$$\int_0^\infty P_{\text{H}_2\text{O}}(t) dt = \Delta Q_{\text{H}_2\text{O}}.$$

From this a normalized instrument response function, $R(t)$, can be defined as

$$R_{\text{inst}}(t) = \left(-\frac{k_1 \cdot k_2}{k_1 + k_2} \right) (\exp(-k_1 t) - \exp(-k_2 t)) \quad t \geq 0$$

$$R_{\text{inst}}(t) = 0 \quad t < 0 \quad (16)$$

$$\int_0^\infty R_{\text{inst}}(t) dt = 1.$$

This instrument-response function describes all water samples and lipid dispersions outside the chain-melting regime. A fit to the water response is given in Fig. 4. For our experimental

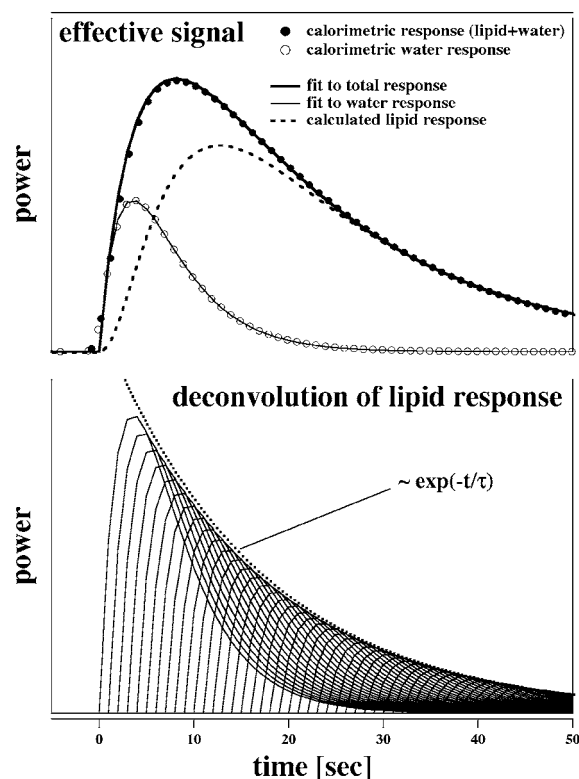
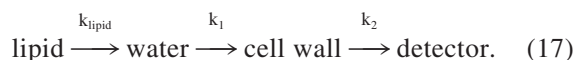


FIGURE 4 Convolution of a calorimetric response signal. *Top*: Response of the lipid dispersion at 23.69°C (filled symbols represent experimental data, the thick line is the fit to the signal according to the deconvolution in the text). Also given is the pure water response (same sample below the transition temperature at 23.08°C (open symbols represent the experimental data, the thin line is the fit)). *Bottom*: Schematic picture of the deconvolution of the lipid response. It is assumed that the heat is released from the lipid membranes with a single exponential behavior. Each released heat quantum results in a calorimetric signal similar to the water response function. The total lipid response thus is a superposition of exponentially decaying water signals (*top panel*, dashed line).

setup, we found that $k_1 = 0.367$ and $k_2 = 0.206$, the latter value corresponding to the calorimetric time constant of ~ 5 s indicated by the manufacturer of the calorimeter.

If a DMPC-MLV lipid dispersion is investigated close to the chain-melting temperature, the power pulse is different from the pure water response. Figure 3 (*bottom right*) displays a trace from the same sample as in the bottom left panel at a slightly different temperature, $T = 23.65^\circ\text{C}$. At this temperature, the sample is in the gel state at 41 bar, but is right in the transition regime at 1-bar pressure. Thus, upon a release of pressure, the lipid system jumps right into the melting regime and heat is absorbed by the sample. Therefore, the overall heat in the power pulse increases, and the time behavior of this pulse is now dominated by the relaxation time of the lipid sample, described by the scheme;



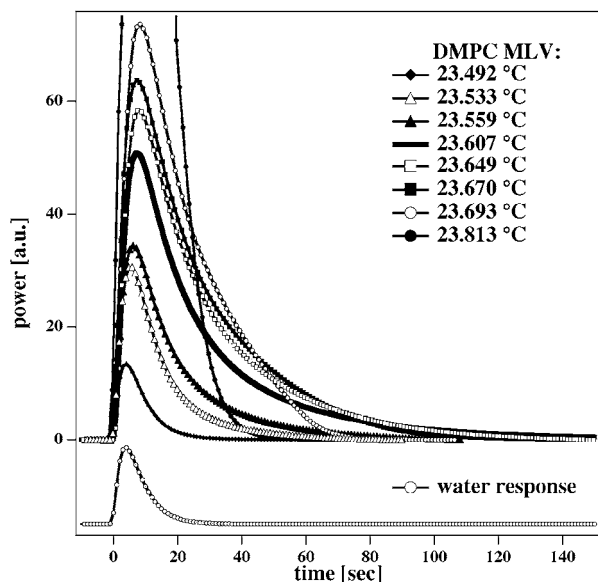


FIGURE 5 Relaxation peaks for DMPC MLV: series of pressure jumps at various temperatures around the heat-capacity maximum. The slowest relaxation time is found at 23.607°C. Relaxation times are shorter below and above this temperature. The integrated area of each peak is a measure for the difference in enthalpy of the system before and after the pressure jump at this temperature.

Let us assume that the uptake or release of heat from the lipid into the aqueous buffer is single exponential,

$$P_{\text{lipid}}(t) = P_{\text{lipid}}^0 \exp(-k_{\text{lipid}}t) \quad t \geq 0$$

$$P_{\text{lipid}}(t) = 0 \quad t < 0 \quad (18)$$

$$\int_0^{\infty} P_{\text{lipid}}(t) dt = \Delta Q_{\text{lipid}}.$$

ΔQ_{lipid} is equivalent to the enthalpy difference of the lipid dispersion before and after the pressure jump. The instrument response of a lipid dispersion (i.e., the recorded signal) is then given by

$$P_{\text{exp}}(t) = \int_{\tau=0}^t P_{\text{lipid}}(\tau) \cdot R_{\text{instr}}(t - \tau) d\tau + P_{\text{H}_2\text{O}}(t), \quad (19)$$

composed as the sum of the lipid response convoluted with the instrument response, plus the pure water response (Fig. 4). The time resolution of our relaxation experiment is enhanced by our deconvolution procedure to ~ 1 – 2 s as compared to the calorimetric instrument response time of ~ 5 s.

Figure 3 (*bottom*) shows that, after the pressure pulse, the temperature in the cell stays constant within a few thousandths of a degree. Thus, the state of the system (which is a function of the temperature) directly after the pulse is well defined.

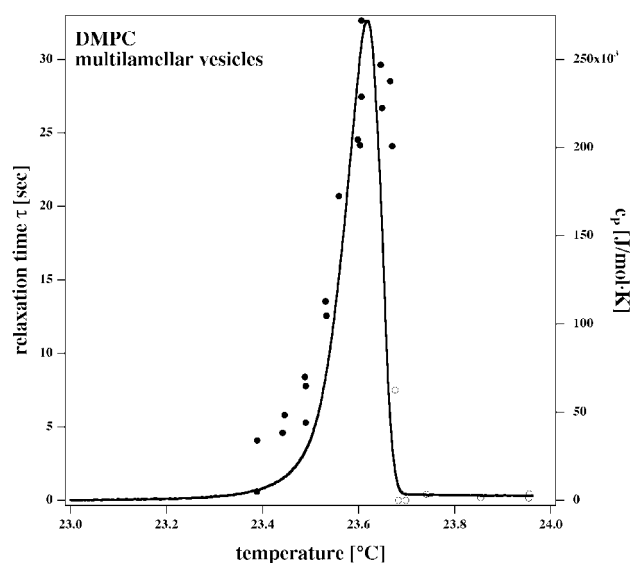


FIGURE 6 Relaxation times of DMPC MLV as a function of temperature superimposed with the heat-capacity profile yield a proportional relationship between τ and c_p . Filled circles were obtained with -40 -bar pressure jumps; open circles with $+40$ -bar pressure jumps.

The shape and amplitude of the power pulse as a function of temperature is shown in Fig. 5. It can be seen that the total area of the pulse (equivalent to the total heat that is absorbed) increases progressively with increasing temperature. It furthermore can be seen that the heat absorption is fast at low temperature, is slowest close to the phase transition temperature, and is fast again above T_m .

Relaxation times of multilamellar vesicles

Multilamellar vesicles display very cooperative transitions with high heat capacity at T_m . The c_p -maximum has a value of ~ 400 kJ/mol. Thus, it can be expected that relaxation rates are especially slow. Figure 6 shows the heat capacity and the relaxation times of DMPC MLV superimposed. It can be seen that the two functions display a very similar temperature dependence and that the proportionality predicted by the linear nonequilibrium model is within error correct. The maximum relaxation time close to the heat capacity maximum is ~ 35 s. The data shown in Fig. 5 originate from two sets of experiments. Solid circles represent pressure jumps from 41 bar to 1 bar, whereas open circles represent pressure jumps from 1 bar to 41 bar. Because the heat capacity profile at 41 bar is shifted by about 0.93 K, relaxation data from the positive pressure jump experiments in Fig. 6 where shifted by -0.93 K. The proportional constant between relaxation time and heat capacity in this experiment is $\alpha = 1.20 \times 10^{-4}$ s·mol·K/J, corresponding to a phenomenological coefficient of $L = 1.8 \times 10^{12}$ J²K/mol²s. The values for α and L are summarized in Table 1.

TABLE 1 The relaxation time, $\tau = (RT^3/L)c_p \equiv \alpha c_p$, for four different lipid preparations, the phenomenological coefficient, L , and proportionality constant, α

Lipid	Phenomenological Coefficient L ($10^{12} \text{ J}^2\text{K/mol}^2\cdot\text{s}$)	α ($10^{-4} \text{ s}\cdot\text{mol/J}$)
DMPC MLV	1.8	1.20
DPPC MLV	2.2; (3.5)	1.17; (0.74)
DPPC LUV	(3.7)	(0.71)
DPPC: Cholesterol = 99:1 MLV	2.18	1.18

L and α were determined at the heat capacity maximum.

The difference of the two values for DPPC, which differ by $\sim 35\%$, probably arises from time-dependent changes in the heat-capacity profiles, which broaden by up to 30% after one week due to slow swelling of the sample.

Uncertain values are given in brackets.

We also measured the relaxation times and heat capacities of multilamellar vesicles of DPPC (Fig. 7). Similarly, we find that the relaxation times change with temperature in a manner proportional to the heat capacity. In two series of experiments, DPPC MLV displayed maximum relaxation rates of 45 and 30 s, respectively. The two different relaxation-time values stem from two independent experiments with different sample preparations. We did not measure the heat capacity profile again in the second experiment. The c_p profile in Fig. 7 (right) was taken from the experiment in Fig. 7 (left) and is therefore indicated as a dashed line

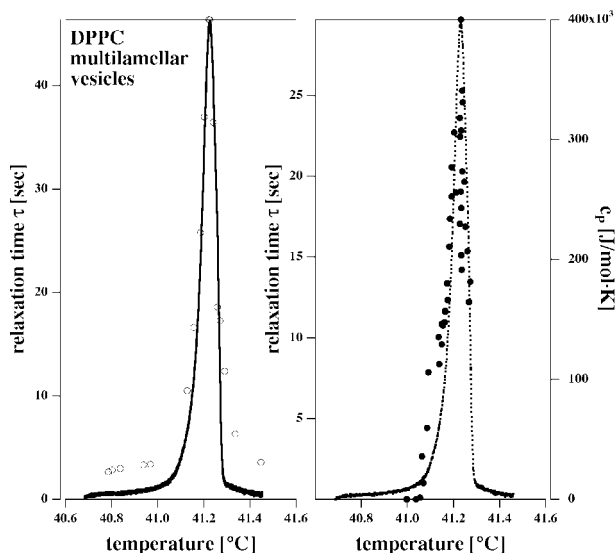


FIGURE 7 Relaxation times of DPPC MLV as a function of temperature superimposed with the heat capacity profile for two independent experiments yield a proportional relationship between τ and c_p . Data were obtained with -40 -bar pressure jumps. The two experiments yield slightly different maximum relaxation times, probably due to slow swelling of the sample that leads to a broadening of the c_p profile by up to 30%. The heat capacity in the right-hand panel was not measured independently. The heat capacity trace given is that of the experiment in the left-hand panel and is indicated as a dashed line.

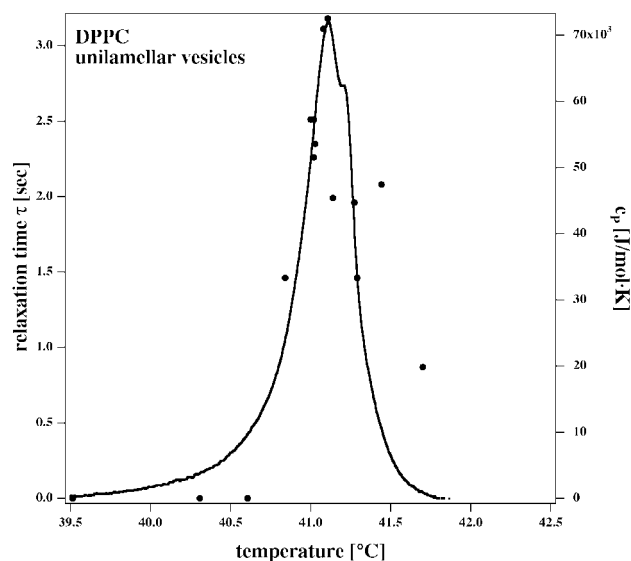


FIGURE 8 Relaxation times of DPPC LUV as a function of temperature superimposed with the corresponding heat-capacity profile. Data were obtained with -40 -bar pressure jumps. Similar to the relaxation time, τ , the value of the heat capacity maximum is one order of magnitude lower value as compared to MLVs.

because it originates from a different sample. Because the two relaxation experiments were partially performed over several days, we assume that slow swelling of the multilamellar sample, accompanied by a broadening of the heat capacity profile (which we did not measure for the experiment in Fig. 7 (right), leads to the discrepancy in absolute relaxation times. With DMPC MLV, we observed a reduction in the heat capacity maximum by $\sim 20\%$ during one week. For the two DPPC MLV experiments, we determined proportional constants $\alpha = 1.17 \times 10^{-4} \text{ s}\cdot\text{mol}\cdot\text{K/J}$ and $\alpha = 0.74 \times 10^{-4} \text{ s}\cdot\text{mol}\cdot\text{K/J}$, corresponding to phenomenological exponents of $L = 2.2 \times 10^{12} \text{ J}^2\text{K/mol}^2$ and $L = 3.5 \times 10^{12} \text{ J}^2\text{K/mol}^2$. It is likely that the absolute value for α of the second DPPC MLV experiment is underestimated due to the reasons given above.

Relaxation times of unilamellar vesicles

We further performed experiments with extruded LUVs of DPPC. Heat-capacity profiles of such vesicles are significantly broadened as compared to multilamellar vesicles. This is probably due to curvature effects (Ivanova and Heimburg, 2001) and the lack of interlamellar confinement (Heimburg, 2000). Assuming similar phenomenological coefficients as in the multilamellar systems, one would expect a maximum relaxation time that is one order of magnitude lower than that found in MLV. This is indeed what we find. The maximum relaxation time we found was ~ 3.2 s (Fig. 8). The error in these data is much higher because our time resolution is ~ 1 – 2 s. Thus, the DPPC LUV relaxation times

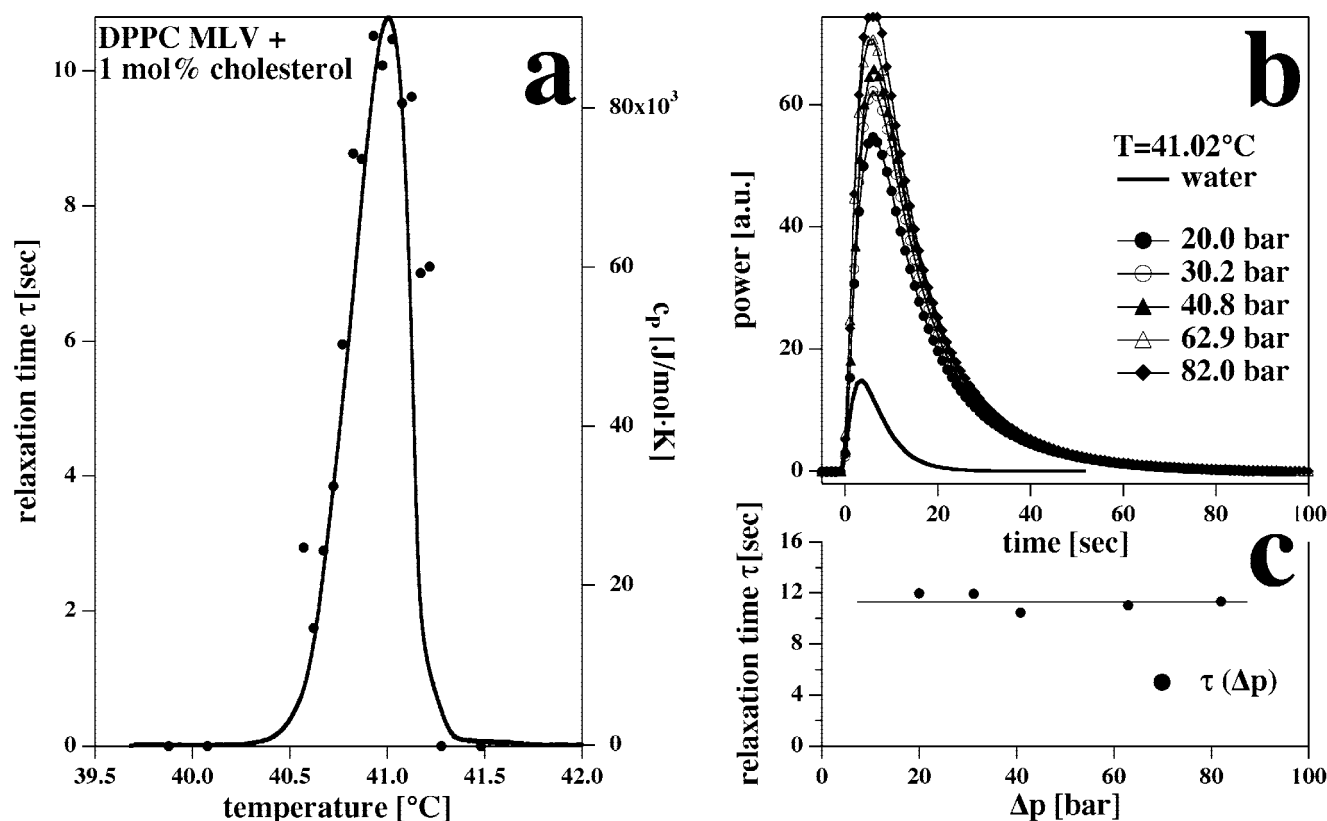


FIGURE 9 Relaxation kinetics of DPPC MLV in the presence of 1 mol% cholesterol. (a) Relaxation times compared to the heat capacity. The heat capacity is smaller by a factor of 4 as compared to DPPC MLV in the absence of cholesterol (Fig. 7 a). Similarly, the relaxation times are shorter to the same degree. (b) Relaxation kinetics at the heat-capacity maximum for pressure jumps of different magnitude ($\Delta p = -20, -30.2, -40.8, -62.9$, and -82 bar). The relaxation kinetics is unaffected by the magnitude of the jump, whereas the amplitude of the response alters slightly. This is caused by the different enthalpies of the membranes at the different starting pressures. (c) Relaxation times for the different pressure jumps shown in panel b are independent of the magnitude of the pressure jump.

are subject to relatively large uncertainty. However, taking the results as they are, we obtain a proportional constants $\alpha = 0.71 \times 10^{-4} \text{ sec}\cdot\text{mol}\cdot\text{K}/\text{J}$, equivalent to a phenomenological coefficient of $L = 3.7 \times 10^{12} \text{ J}^2\text{K}/\text{mol}^2$. This value is very similar to one of the DPPC MLV experiments.

Relaxation times in the presence of cholesterol

We also studied the relaxation kinetics of a simple lipid mixture (Fig. 9 a). Upon addition of 1 mol% of cholesterol to DPPC MLV, the heat capacity at the maximum is decreased by about a factor of 4. Also, the maximum is slightly shifted to lower temperatures by ~ -0.2 K. When analyzing the relaxation times of these mixtures, they also decrease to a maximum value of ~ 10.5 s as compared to the pure DPPC MLV with ~ 30 – 40 s (Fig. 7 (left)). This results in a proportional constant of $\alpha = 1.18 \times 10^{-4} \text{ sec}\cdot\text{mol}\cdot\text{K}/\text{J}$, equivalent to a phenomenological coefficient of $L = 2.18 \times 10^{12} \text{ J}^2\text{K}/\text{mol}^2$, which is very similar to the values obtained for DMPC MLV and DPPC MLV. Thus, cholesterol reduces the relaxation time to the same degree as it lowers the heat capacity. This supports our view that the principles on

the relation between heat capacity and relaxation times outlined above may be of general character.

One of the assumptions from the Theory section was that the relaxation times depend only on the final state of the systems but not on the state before the pressure jump. This is also the result of applying pressure jumps of different magnitude (from -20 to -82 bar) to the DPPC/cholesterol system at the heat capacity maximum (Fig. 9 b). While the target state remains unchanged ($p = 1$ bar, $T = 41.02^\circ\text{C}$), the state before the pressure jump changes due to different starting pressures. This affects the overall amplitude of the power pulse, but does not change the relaxation kinetics. Relaxation times are independent of the magnitude of the pressure jump (Fig. 9 c).

DISCUSSION

In this paper, we investigated relaxation processes of lipid membranes by pressure-jump calorimetry. We provided a theoretical analysis of relaxation processes based on linear nonequilibrium thermodynamics concepts. A model of such

processes is not available in the literature, although the idea of relating thermodynamic forces to heat capacities in the melting processes of biological systems has been brought up before by E. Freire's group (van Osdol et al., 1991b). The principle outcome of the model is that relaxation times of lipid vesicles are proportional to the heat capacity close to chain-melting events. This prediction has been verified in Monte Carlo simulations. Experimentally, we found relaxation times in the range of up to 45 s in multilamellar systems, which are extremely cooperative and therefore display a very pronounced and narrow heat capacity maximum. The proportional relation between the relaxation time and heat capacity was found to be true within experimental error. A further experiment on extruded unilamellar vesicles confirmed this finding. Extruded vesicles display a much broader melting profile and consequently a lower maximum heat capacity. The relaxation times found for this system therefore were much faster, i.e., in the range of 3 s.

The relaxation process is a cooperative phenomenon close to the melting point. Our theory implies that the main relaxation process is not an activated process with any kind of activation barrier, but rather the result of the large degeneracy of states with similar free energy close to the heat capacity maximum. Our model is therefore based on entropy arguments. The equilibration process is dominated by the mean time that the system needs to undergo a random walk through all the degenerate states surrounding the equilibrium state. Close to the heat-capacity maximum, the degeneracy of states with about equal free energy is maximum, and so is the number of states visited by the system. Our line of argument, therefore, cannot be related to nucleation theory and nucleus growth, but is a completely different and new approach. It is valid as long as the fluctuations are of macroscopic nature (domains) and not on the molecular scale (single lipids). It is possible that events of more local nature (head-group rearrangements, isomerizations, or even volume changes of vesicles not related to domain growth are responsible for the short relaxation processes observed in some of the optical experiments.

Data for relaxation times in the literature suffer from a lack of temperature precision. The temperature accuracy in optical measurements is lower than in calorimetry because windows for light transmission are required, which are open to the environment and not thermostated. This usually results in temperature gradients. The peak width in multilamellar vesicles, however, is less than 0.1 K. For this reason, relaxation data in the literature have smaller numerical values as reported here. Tsong and Kanehisa (1977), who probably published the first paper in this field, found relaxation times in the range of 2 s plus a fast process of about 30 ms from turbidity measurements of DMPC MLV after a temperature jump. In other experiments, maximum relaxation times of 120 ms have been found (Gruenewald, 1982). Elamrani and Blume (1983), by similar means, found a slow

relaxation process in the range of up to 3 s for dilauroyl-, dimyristoyl-, and ditetradecyl phosphatidic acid. Furthermore, they described two faster processes at 100 and 10 ms. Another study on dimyristoyl phosphatidic acid/cholesterol bilayers yielded comparable results (Blume and Hillmann, 1986). Gruenewald et al. (1980) studied sonicated vesicles of DMPC and DPPC in turbidity measurements after a pressure jump and found relaxation times below 40 ms (sonicated vesicles display very broad melting profiles [Ivanova and Heimburg, 2001] and relatively short time constants are expected). For electrostatically triggered transitions, Strehlow and Jähnig (1981) found relaxation times in the range up to 200 ms. The latter paper attempts to rationalize the relaxation behavior in a nonequilibrium approach by considering a nucleation and growth process. Genz and Holzwarth (1986) found relaxation times in the 20-ms range. A similar timescale was found in the presence of cholesterol (Genz et al., 1986), although fluctuations in cholesterol-containing systems are largely reduced (Halstenberg et al., 1998).

A problem with all the measurements described above is the fact that the temperature is not well defined. According to our model, the relaxation process is determined by the temperature/pressure after the perturbation of the system (this assumption was confirmed by the measurements shown in Fig. 9 b). In optical methods, temperature accuracy is difficult to achieve, because it must be in the range of $1/100$ K, and this is impossible in an optical setup where cuvette windows are exposed to the environment. Usually the temperature accuracy is worse than 0.1 K. Thus, relaxation times much smaller than those found by us may partially be due to the difficulties in adjusting absolute temperatures close to the heat-capacity maximum.

A way out of this dilemma are calorimetric means, mainly explored by R. L. Biltonen's group (Johnson et al., 1983; van Osdol et al., 1989, 1991a, 1992). They investigated relaxation processes in a volume perturbation calorimeter (a comparable approach was made with multifrequency calorimetry to analyze timescales of protein unfolding by van Osdol et al., 1991b). Relaxation times for DPPC MLV of up to 4 s were reported. This is still one order of magnitude faster than in our experiments. This probably is caused by a principal problem of the experimental approach. Although the absolute temperature is very exact, the pressure in these calorimeters oscillates by a value that corresponds to a shift of the transition of ~ 0.1 K. Because the relaxation is a feature of the system in a well-defined state, it is not exactly clear which relaxation is measured in such experiments where the state undergoes a continuous change. The relaxation is probably dominated by the state with the fastest relaxation during the continuous perturbation process. Relaxation times at the maximum cannot be determined, if the perturbation is larger than the half width of the transition. Therefore, at the c_p maximum of DPPC MLV (with a transition halfwidth of less than 0.1 K),

the response of the system is smeared out. Because, in our approach, the experiment is both isothermal and isobaric, we have a very good estimate of the relaxation times at the maximum. However, one may consider the volume calorimeter as complementary to our setup, because it is able to record faster relaxation processes and thus supplies data further away from the melting temperature. Van Osdol et al. (1991a) furthermore found that relaxation times in unilamellar vesicles are significantly shorter (~ 80 ms). Interestingly, they also found a reduction of the relaxation times in the presence of the local anesthetic dibucaine, which correlates with the reduction in the cooperativity of the melting transition (van Osdol et al., 1992). This is in agreement with our model.

The difference in relaxation times in calorimetric methods and those obtained by optical means also poses the question of whether the same relaxation processes are investigated. Calorimetry directly monitors enthalpy changes and therefore changes in lipid state. Optical methods may also record changes in shape and local processes in the environment of fluorescence labels. Thus, in calorimetric methods, it is clearer what feature of the system exactly relaxes. From calorimetric measurements, it is obvious that the predominant part of the enthalpy relaxation (if not all) is a very slow process. No significant contribution of fast processes (which would appear in our experiments as a seemingly increased water response) are required. However, in all optical methods, several time constants are required. We assume that some of them do not directly relate to the cooperative melting process even though they also show maxima in the transition range.

From our experiments, we determined proportionality constants and phenomenological coefficients that were very similar for the different lipid systems (within experimental reproducibility). Thus, one may suspect that similar phenomenological coefficients will be found for other lipid systems. We have shown that, in the presence of cholesterol, the relaxation times change to the same degree as the heat capacity (Fig. 9 *a*). This may allow us to raise a few speculative points on general mixtures and biological membranes. Let us assume that, in mixtures, the relaxation times are generally related to the heat capacity. There are biological systems where melting has been demonstrated. This includes lung surfactant, which displays a heat capacity maximum close to 27°C (Ebel et al., 2001). It consists of a lipid mixture with a high content in DPPC, but also contains two integral proteins, SP-B and SP-C. Lung surfactant has a heat capacity at maximum of ~ 1.6 J/g·K, which is about a factor of 330 smaller than the heat capacity maximum of DPPC MLV (Ebel et al., 2001; Grabitz, 2001). Assuming a similar phenomenological coefficient as for DMPC and DPPC, one obtains fluctuation-related relaxation times in the range of 50–130 ms (a further, slow relaxation process in mixtures may be due to lipid diffusion). This prediction is based on the assumption that lipid domains exist in lung

surfactant at room temperature and that their fluctuations dominate the relaxation behavior. This may serve as an upper estimate of possible relaxation times in biological membranes, because lung surfactant is the most prominent example for collective melting processes in biological membranes. The time scale of 1–100 ms, however, is biologically relevant because many protein transitions happen right in this time regime. Ion release through potassium channels (and other channels) happens in the range of 10 ms (Hille, 1992). This is also the time regime of action potentials. Relaxation measurements on erythrocytes and erythrocyte ghosts yield relaxation times between 0.4 and 9 s (Tsong et al., 1976). In these experiments, it seems difficult to attribute the relaxation process to any specific mechanism (e.g., hemolysis, rupture, etc.). One must, however, also consider the possibility that cooperative melting events as described here contribute to the kinetics.

It is difficult to prove that heat capacity events at physiological conditions of biomembranes are present, because, in calorimetric experiments of most biological membranes, no heat capacity anomalies (maxima) are evident. This does not imply that there are no heat capacity events, but rather that they are difficult to distinguish from the baseline. Most likely, melting events are a continuous process in biological membranes over the whole temperature regime, which is of biological interest because all biological membranes contain lipids with high melting points and also a significant content of cholesterol and proteins, which affect melting behavior. If this were the case, heat capacity events would be smeared out to a significant degree, because biological membranes are quite heterogeneous with hundreds of different lipids that melt at very different temperatures. A further complication is that, in calorimetric experiments, it is difficult or even impossible to distinguish heat-capacity events originating from lipids and from proteins. Erythrocyte membranes, for instance, display a heat-capacity anomaly at body temperature that is most likely linked to a transition in the spectrin network (unpublished data from our lab). Generally, one should therefore conclude that heat capacities in biological membranes are low and that fluctuations do not occur on a global level but rather locally at domain interfaces or in the lipid interface of proteins.

So far, little is known about cooperative processes in biomembranes, and it would be of ultimate interest to measure heat capacities of such systems. Cooperative events in biomembranes are a possible control mechanism, based on changes in compressibilities (Heimburg, 1998) and of time constants. It is hard to believe that nature does not make use of such powerful possibilities that can be understood on a physical basis. At this point, the predictions about relaxation times in biological membranes are a speculative generalization of the relaxation experiments and the modeling presented in this paper.

CONCLUSIONS

In this paper, we present a nonequilibrium thermodynamics approach to describe relaxation processes at cooperative melting transitions in lipid membranes. The predicted proportional relation between relaxation times and heat capacity was confirmed in isothermal pressure jump calorimetry and in computer simulations. This finding may contribute to a deeper understanding of relaxation phenomena in biomembranes.

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