

# The Kinetics of Membrane Phase Transitions

## INTRODUCTION

There is a consensus that the biological membrane consists of a phospholipid bilayer matrix in which various protein components are immersed. Several physical and chemical studies have provided a distinct picture of the time-averaged structure of such membranes. It is also understood that the interactions between the phospholipids and the membrane bound proteins are important in the function and regulation of these proteins. However, most of these ideas are conceived only in terms of a static picture. Little consideration or experimental effort has been put forth toward understanding the temporal aspects of such interactions. There has been little effort, either theoretical or experimental, to describe the cooperative dynamics of the membrane components. It is well known that the phospholipid molecules may diffuse within the plane of a single monolayer of the membrane bilayer or redistribute from one bilayer surface to another, and that proteins can readily diffuse within the membrane.

For some time, the working hypothesis in our laboratory has been that the lipid matrix of a biological membrane exists in a dynamic equilibrium of ordered and disordered clusters of variable size. It is believed that near the phase transition temperature,

which differs for each lipid, the clusters begin to change in size and distribution. If the temperature is decreased a “freezing” of the lipids occurs in which the gel clusters enlarge by fixing neighboring molecules into the more rigid and more compact gel state. As the temperature rises through the phase transition, the matrix melts or relaxes, allowing the liquid-crystalline phase clusters to predominate the structure. We believe that it is the size distribution of these ordered and disordered clusters and their fluctuations in size which confer special properties upon the membrane and are related to regulation of specific protein functions. In order to obtain more information about the dynamics of membrane structure in the phase transition region, a novel volume-perturbation calorimeter was designed to measure the kinetics of lipid phase transitions.

The traditional methods used to determine kinetic parameters of macromolecular systems at presumed equilibrium include perturbation techniques such as temperature and pressure variations or stopped-flow methods which alter the relative concentration of the various components. Generally, the resulting temporal change in the equilibrium position is monitored using an optical observable. In some biochemical systems such as nucleic acids and proteins, these approaches have been employed successfully. However, in phospholipid systems the success has been limited by the lack of a sensitive natural optical probe and the difficulty in interpreting the existence of multiple relaxation processes. For example, the kinetics of the main transitions of dimyristoyl- and dipalmitoyl-phosphatidylcholine (DMPC and DPPC)\* dispersions have been studied using temperature jump techniques and monitoring changes in turbidity. It was discovered that the relaxation process is complex, and at least two short relaxation times exist:  $\sim 1$  s and 0.01 s.<sup>1</sup> These data were difficult to interpret for two reasons. Either the observable quantity was difficult to interpret in terms of its structural basis, or there were alterations in the membrane system caused by the method used to monitor or induce

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\*Abbreviations used: DMPC, dimyristoyl-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine;  $t_m$ , melting temperature in °C;  $\Delta T_{1/2}$ , half-width of phase transition;  $\phi C_p$ , excess heat capacity; DSC, differential scanning calorimetry;  $K_T$ , isothermal compressibility.

the changes in the equilibrium position. For example, it is difficult to determine the nature of physical changes in a membrane when turbidity is monitored because the dimensions of the membrane are significantly larger than the wavelength of the monitoring light. Also, the voltage induced temperature jump technique can produce structural changes in membrane systems.<sup>1</sup> A number of investigators have employed the fluorescence properties of hydrophobic dyes to monitor changes in membrane structure.<sup>2</sup> However, in equilibrium experiments, such as differential scanning calorimetry, the addition of minor contaminants, such as these fluorescence probes, may alter the thermodynamic behavior of the system during the phase transition.

In order to investigate the dynamics of cluster formation within the phase transition region we needed to obtain kinetic data about the phase-like transitions of membranes that could be directly compared with equilibrium scanning calorimeter measurements. Furthermore, we wished to use the intrinsic thermodynamic properties of the phospholipid matrix itself rather than attempting to interpret a secondary indicator, such as a fluorescent probe which could alter the properties of the membrane system. In addition, it is essential that the perturbations from equilibrium be small, reversible and nondestructive. Toward this end we constructed a volume-perturbation instrument<sup>3</sup> based on the original design of Clegg *et al.*,<sup>4</sup> as modified by Halvorson.<sup>5</sup> It monitors the temperature and pressure changes, which indicate temporal changes in the equilibrium state of the system, induced by a forced volume change. This approach is based upon the large volume changes associated with the phase transitions of model multilamellar and single lamellar vesicles made from neutral phospholipids.

## BASIS OF OUR APPROACH

In our experimental approach, the voltage-dependent extension of a stack of piezoelectric crystals is used to force the sample solution to undergo a small, adiabatic, bidirectional and periodic volume change. The pressure and temperature changes induced by the volume changes are the driving forces which alter the equilibrium point of the gel to liquid-crystalline transition in a small, periodic

fashion. From the comparison of the experimentally observed temperature and pressure waveforms with the driving waveform, that is, the volume change, we can deduce the kinetic and thermodynamic properties of the sample solution.

The main phase transition melting temperatures,  $t_m$ , for multilamellar DMPC and DPPC liposomes have been shown to have a strong dependence on the hydrostatic pressure.<sup>6,7</sup> The relative excess heat capacity,  $\phi C_p$ , for DPPC liposomes, as measured by differential scanning calorimetry at different applied pressures of helium are shown in Fig. 1.<sup>7</sup> Increasing the hydrostatic pressure increases the melting temperature for both the low- and high-temperature transitions by approximately 0.024 °C/atm, but does not alter the enthalpy change, i.e., the integral of the heat capacity curve, or the shape of the transition profile. It should be noted that the phase transition half-width,  $\Delta t_{1/2}$ , is a sensitive indicator

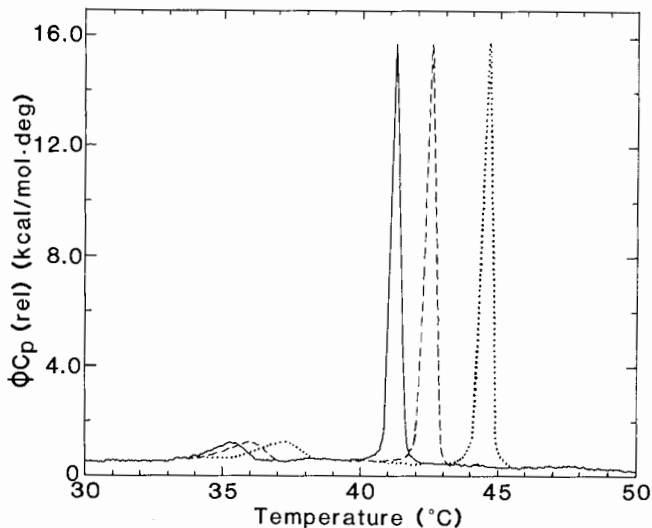


FIGURE 1 Relative heat capacity ( $\phi C_p$ ) of a multilamellar dispersion of DPPC in 0.05 M KCl at a pressure of 1 atm (—) or helium pressures of 68 (----) and 136 atm (.....). Increasing the pressure increased the  $t_m$  for both low and high temperature transitions by  $\sim 0.024$  °C/atm, but did not alter either the enthalpy change or the shape of the transition profile. This shows that the  $t_m$ ,  $\Delta t_{1/2}$ , and enthalpy are independent of pressure, and the pressure change does not alter the cooperativity of the system. [Redrawn from Mountcastle *et al.* (Ref. 7).]

of the degree of cooperativity of the phase transition. Since the shape of the phase transition is unaltered, the degree of cooperativity is not affected by pressure.

An example of the cyclic nature of our instrument can be easily described by reference to the work of Mountcastle *et al.*<sup>7</sup> as redrawn in Fig. 1. Initially, this DPPC solution is equilibrated at 1 atm and 41.8 °C which corresponds to a state of the lipid matrix about 95% of the way through the phase transition, as shown by the solid line in Fig. 1. We now decrease the volume of the sample compartment by an amount sufficient to increase the pressure to 68 atm, in approximately 1 ms. At equilibrium at this temperature and pressure, only about 5% of the lipids exist in the gel state, as shown by the dashed line in Fig. 1. As the lipids freeze to the gel phase, our instrument monitors the heat released, as measured by the temperature of the solution. After sufficient time has elapsed for the system to achieve its new equilibrium position, the sample compartment volume is returned to its original starting volume, and our instrument monitors the absorption of heat as the liposomes melt while returning to their initial liquid-crystalline state. In an actual experiment, the situation is more complex than presented so far since the lipid also undergoes a microscopic volume change during the phase transition. Thus, as a consequence of a forced volume change of the sample compartment, the lipids will decay to a new equilibrium position along a kinetic path involving changes in both heat released and a microscopic volume change. The possibility for changes in microscopic volume implies that the pressure induced by a fixed volume change of the sample compartment need not be constant. We therefore alter the sample compartment volume in a known periodic manner and simultaneously monitor both temperature and pressure as a function of time.

In normal operation of the instrument, the forced volume change only induces a change in pressure of approximately 4 atm which forces the liposomes through only a small portion of the phase transition. This situation is shown diagrammatically in Fig. 2. In this simulated example, a solution was equilibrated at 41.0 °C and 8 atm pressure. Then the volume was altered to generate a 4 atm pressure change, equivalent to a temperature change of 0.1 °C. Operating at such low volume changes means that: (1) our instru-

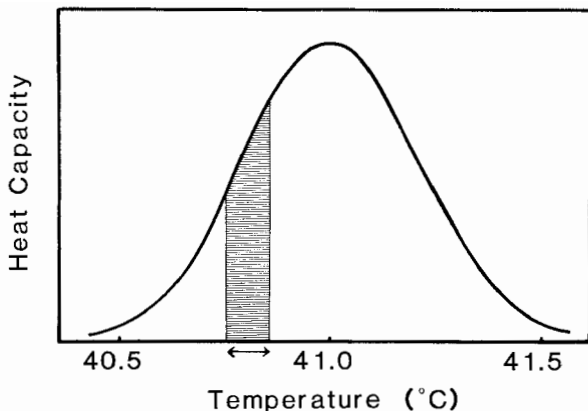


FIGURE 2 A simulated excess heat capacity curve for DPPC liposomes showing the portion of the phase transition, shaded area, induced by a 4 atm pressure change. Our instrument makes it possible to measure the kinetics of the phase transition at varying stages within the transition by inducing small step-like perturbations in the system.

ment truly makes a small, reversible perturbation from equilibrium in a nondestructive manner, and (2) these small perturbations allow the measurement of the kinetics of the phase transition at varying stages within the phase transition. The disadvantage of working with such small volume perturbations is that the resulting time-dependent temperature and pressure changes are smaller. The shaded area of Fig. 2 corresponds to the fraction of the total enthalpy and volume changes which are induced by a 4 atm pressure change. A 4 atm pressure change can be generated by a fractional volume change of  $1.8 \times 10^{-4}$  in water.

The phase transition temperatures for multilamellar DMPC and DPPC liposomes have been shown to vary by  $0.024 \text{ }^\circ\text{C/atm}$  of pressure.<sup>7</sup> Consequently, the effect of a 4 atm pressure change will be a  $0.1 \text{ }^\circ\text{C}$  change in the melting point of the lipids. Since the half-width of the DMPC transition is only approximately  $0.2 \text{ }^\circ\text{C}$ , the application of a sufficient volume change to induce a 4 atm pressure change can shift the equilibrium sufficiently to induce as much as 40% of the lipids to shift from their liquid-crystalline to gel state. This implies that a 4 atm increase in pressure on a 0.1 M DMPC solution will raise the temperature by as much as  $0.2 \text{ }^\circ\text{C}$

since the enthalpy change for the main DMPC transition is approximately 5.0 kcal/mol. This is a maximal estimate since it is calculated from the steepest portion of the transition. It is also a maximal estimate since, when this shift in equilibrium position is accomplished under adiabatic conditions, the lipid will release heat and raise the solution temperature, thus partially compensating for the pressure change.

The basic experimental approach outlined has a number of distinct advantages over previous methods which have been employed to measure the kinetics of lipid phase transitions. First, the perturbations from equilibrium are small and reversible. Second, the method of perturbing the equilibrium, i.e., the sample compartment volume change, is homogeneous throughout the solution being studied. Third, we are directly measuring thermodynamic state variables of the solution, temperature and pressure, which can be related to the thermodynamic parameters of the solute; e.g., enthalpy, excess heat capacity and volume changes. Fourth, this method of monitoring the temporal changes in the equilibrium position does not alter the solute properties. Other techniques, such as the addition of a fluorescent dye can have large effects on the properties of the solute. Fifth, this apparatus can be used in a "single pulse" mode similar to the temperature jump technique but it has the advantage that it is bidirectional. Sixth, the apparatus can be used with a periodic forcing function in a manner analogous to phase modulation fluorometry and as a consequence allows the experimental data to be signal averaged. Seventh, this experimental approach allows the measurement of the kinetics of heat release and volume changes over a small portion of the phase transition, whereas most previous techniques required the lipids to pass through the entire transition region.

## DESCRIPTION OF THE VOLUME-PERTURBATION APPARATUS

In this instrument, a stack of piezoelectric crystals, located to the right of driving piston E in Fig. 3, expand when a voltage is applied, forcing the driving piston, E, against the Mylar diaphragm, N, of the sample compartment. This produces a small adiabatic, bidirectional volume change in the sample solution. The sample com-

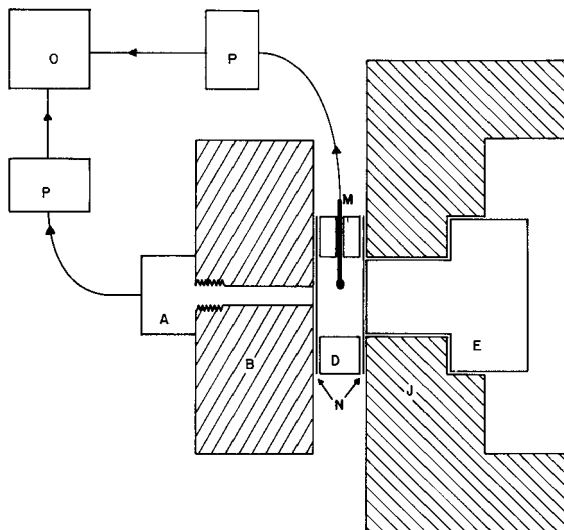


FIGURE 3 Diagram of the volume jump cell: (A) pressure transducer, (B) sample compartment support, (D) sample compartment ring, (E) driving piston, (J) main cell body, (M) temperature transducer, (N) Mylar diaphragm, (O) computer, and (P) signal amplifier. For a complete description, see text section entitled "Description of the Volume-Perturbation Apparatus." [Redrawn with permission from Johnson *et al.* (Ref. 3).]

partment (0.8 ml volume) is a right circular stainless steel cylinder 13 mm in diameter and 6 mm thick (D, in Fig. 3). A high-speed thermistor (M, in Fig. 3) was placed in the compartment through a threaded hole drilled in the sample compartment ring. The choice of a thermistor and its location were dictated by our desire to have a fast time response. Another transducer, which is located directly opposite the driving position on the other side of the sample compartment (A, in Fig. 3), monitors pressure changes through a second Mylar diaphragm. Temperature control is maintained by a water bath with remote temperature sensing. The set point of this bath can be externally programmed by the computer (O, in Fig. 3) to stabilize the calorimeter to better than 0.01 °C. Water is circulated through temperature regulation copper coils soldered to the cell body, J, and the whole cell is contained in an insulated compartment.

In our apparatus, a pressure increment of about 5 atm is gen-

erated by a change in voltage of approximately 3/4 to 1 kV on the crystals. We have employed both sine and square wave driving functions for the volume. A square wave is advantageous for frequency domain (Fourier) analysis since it contains multiple odd numbered harmonics of the driving frequency. This allows us to collect data relating to multiple frequency perturbations from a single experiment.

The signal from the 10 k $\Omega$  thermistor (M, in Fig. 3), a Thermometrics, Inc. (Edison, N.J.) FP07DA103N is processed with two different amplifiers. The first has a fixed-resistance Wheatstone bridge and is used to produce a signal directly proportional to the temperature of the cell. The second amplifier has a constant gain of 113 over the temperature range 20–45 °C and a variable input offset. By nulling the offset in the second high-gain amplifier, a very accurate relative measurement of the time-dependent temperature change is obtained. The signal from the piezoelectric pressure transducer (A, in Fig. 3), a Kulite Semiconductors (Ridgefield, N.J.) XTM-1-190-500 with an internal fixed Wheatstone bridge, is amplified in an analogous fashion. Both transducers are excited by separate voltage regulators set at 2 V. Signal-averaging techniques are usually employed to improve the signal-to-noise ratio, which increases as the square root of the number of cycles averaged.

We are currently modifying this instrument in a number of ways. We have replaced the external function generator, used to generate the voltage waveform for the piezoelectric crystals, by a computer generated waveform. While this modification will not improve the data *per se*, it does improve the ease of operation of the instrument. It also increases the functionality of the instrument, since it will allow the volume changes to be programmed by any arbitrary waveform which might be desired.

Another modification has been to utilize a 12 channel, simultaneous, direct memory access, analog to digital converter (DT3388; Data Translation; Marlborough, Mass.). The ability to collect the data from multiple inputs simultaneously, rather than serially as with conventional multiplexed analog to digital converters, makes the interpretation of the phase shift of Fourier components easier.

The last, and most significant, modification of the instrument will be the addition of optical components. The instrument cur-

rently being constructed will, in addition to temperature and pressure, also measure turbidity, light scattering and fluorescence. This will enable us to correlate changes in these optical properties with changes in thermodynamic properties and to correlate our experimental results with those obtained by other workers. This also makes the study of protein lipid interactions practical with this instrument.

## TYPICAL RESULTS

Assuming that the system is adiabatic and that only pressure-volume work is done on or by the system, one can derive the relationship

$$\left. \frac{\partial T}{\partial P} \right|_s = TV\alpha/C_p \quad (1)$$

where  $\alpha$  is the coefficient of thermal expansion,  $(1/V) (\partial V/\partial T)_p$ , at constant pressure.<sup>5</sup> Evaluating this expression for water at 20 °C and 1 atm yields  $1.6 \times 10^{-3}$  °C/atm. An experimentally observed value of  $\Delta T/\Delta P = 1.5 \times 10^{-3}$  °C/atm was measured with our instrument for calibration purposes (data not shown). A least-squares analysis of the data, assuming a single exponential decay process, yielded a thermal response time for the instrument of 3 ms. In a similar manner, the response time for the application of the voltage to the crystal stack and the resulting volume change was determined to be 1–2 ms. Since the sample solution is in direct contact with the cell body, it is expected that any induced temperature difference will be rapidly dissipated. This dissipation can be observed and its characteristic decay time measured when the apparatus is operated at 0.01 Hz. A least-squares analysis of such an experiment (data not shown) yields an apparent decay time constant for heat transfer from the sample to the environment of approximately 30 s. The combination of the thermal response time and the thermal dissipation time indicates that this instrument is functional in a time domain which is not obtainable by other methods which are usually applied to the study of membrane transitions.

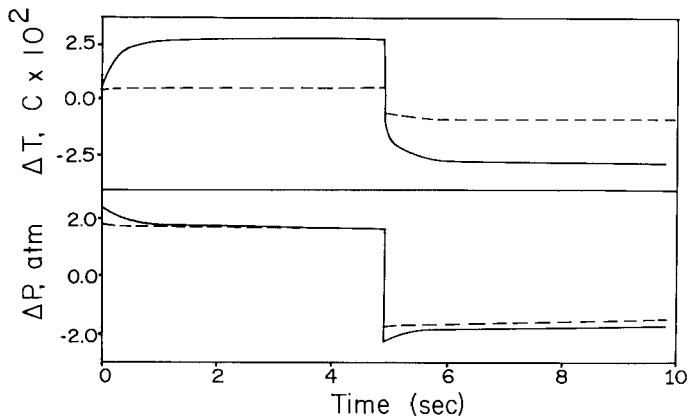


FIGURE 4 Response characteristics of multilamellar DMPC vesicles. The solid line represents a pressure perturbation of 4 atm applied at 24.15 °C which forces the system partially through the gel to liquid-crystalline transition. The dashed line shows a similar perturbation, for comparison, at 22.85 °C, outside of the phase transition region. Conditions: 0.2 M DMPC; 15% sucrose; 0.05 M N-tris-[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES buffer); 0.05 M KCl; 0.001 M Na azide; pH 7.4; average pressure, 4 atm. [Redrawn with permission from Johnson *et al.* (Ref. 3).]

The use of the instrument for a more interesting experimental system is shown in Fig. 4, where we present the results with a dispersion of multilamellar vesicles of DMPC: 0.2 M, in 0.05 M N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, No. T-1375, Sigma Chemical Co., St. Louis, Mo.) buffer, 0.05 M KCl, 0.001 M Na azide, pH 7.4. The lipid was obtained as a chloroform suspension from Avanti Polar-Lipids, Inc. (Birmingham, Ala.). The lipid was dried under vacuum, and the buffer was then added to the dried lipid with vortexing at 30 °C. This procedure yields multilamellar liposomes with a broad size distribution. The buffer also contained 15% sucrose to prevent the vesicles from sedimenting in the cell during the course of the experiment.<sup>2</sup> The solid line in Fig. 4 corresponds to a pressure perturbation of 4 atm applied at 24.15 °C, thereby forcing the system partially through the gel to liquid-crystalline transition. For comparison, the dashed line represents a similar perturbation of 4 atm pressure at 22.85 °C, outside of the phase-transition region. At both of these temperatures, a static pressure of 6 atm was applied and the cor-

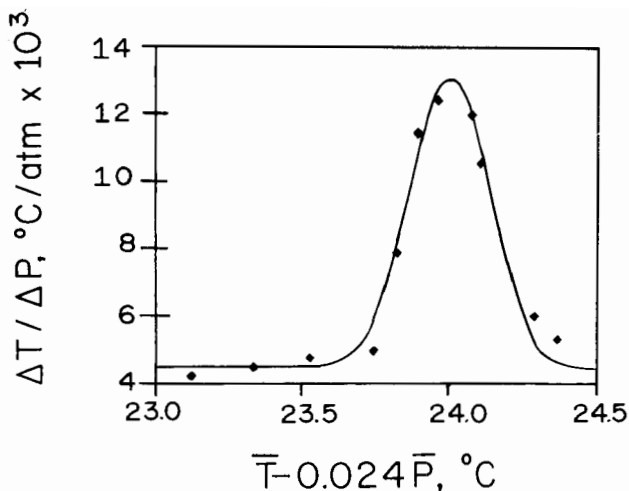


FIGURE 5 Excess heat capacity curve determined by differential scanning calorimetry (solid line) and the response of our instrument (data points) with the same multilamellar DMPC vesicle solution described in Fig. 4. The abscissa is the pressure-induced temperature change plotted against the "apparent temperature" corrected for variations in ambient pressure. Volume perturbation was at 1.0 Hz.

responding melting temperature of the lipid is approximately 24.1 °C with a half-width of approximately 0.2 °C as measured by differential scanning calorimetry (see Fig. 5). In comparing the two curves in Fig. 4, note both the increase in  $\Delta T/\Delta P$  ( $1.5 \times 10^{-2}$  °C/atm at 24.15 °C versus  $3.4 \times 10^{-3}$  °C/atm at 22.85 °C) and the slower time response of the temperature changes in the transition region. The estimated relaxation time for the lipid is approximately 2 s, which is in reasonable agreement with the results of Yager and Peticolas.<sup>8</sup> It should be noted that the relaxation process is only approximately a single exponential and that the estimated relaxation time is dependent on exactly where in the transition range the experiment is performed. At 24.15 °C, the rapid 1/2 atm decrease in pressure after the application of the pressure pulse corresponds to a volume reduction in the lipids as they reorder to decrease the stress on the system. Also note that the time response of the thermistor, approximately 0.003 s, is negligible when compared to the response time of the lipids in the phase-transition region.

A figure analogous to an excess heat capacity curve determined with our prototype instrument is shown in Fig. 5. In this figure, the abscissa is the pressure-induced temperature change per atmosphere, while the ordinate is an “apparent” temperature corrected for the variations in ambient pressure. Comparing the data in Fig. 5 with that obtained by differential scanning calorimetry (data not shown) yields melting temperatures which differ by 0.015 °C and provide good agreement for the half-width of the transition.

It is particularly noteworthy that the half-width as measured by our apparatus is in agreement with that measured by differential scanning calorimetry, DSC. We know from an analysis of the half-width of the DSC results that the phase transition occurs in a cooperative fashion involving clusters of hundreds of phospholipid molecules. The similarity in half-widths observed with our instrument indicates that the kinetics we are observing also involve phospholipid clusters of hundreds of molecules.

These data may be analyzed by a number of methods. First, the data may be analyzed by least-squares fitting to multiple exponential decay rates. Second, the data may be analyzed by least-squares fitting to the numerical solutions of systems of differential equations which describe the instrument and possible molecular interactions of the sample being studied. The data can also be analyzed by the use of Fourier methods. It has previously been shown<sup>4,5,9</sup> that the frequency dependence of the response for an instrument of this type can be related to the characteristic time constants of the chemical reactions as:

$$\frac{d(\text{Temperature})}{d(\text{Pressure})} = \sum \varepsilon_i \quad (2)$$

$$\varepsilon_i = \frac{\text{Amp}_i}{[1 + (\omega\tau_i)^2]^{1/2}} \quad (3)$$

$$\text{Tan}[\Psi] = \frac{\sum \varepsilon_i \text{Sin}(\Psi_i)}{\sum \varepsilon_i \text{Cos}(\Psi_i)} \quad (4)$$

$$\text{Tan}[\Psi_i] = \omega\tau_i \quad (5)$$

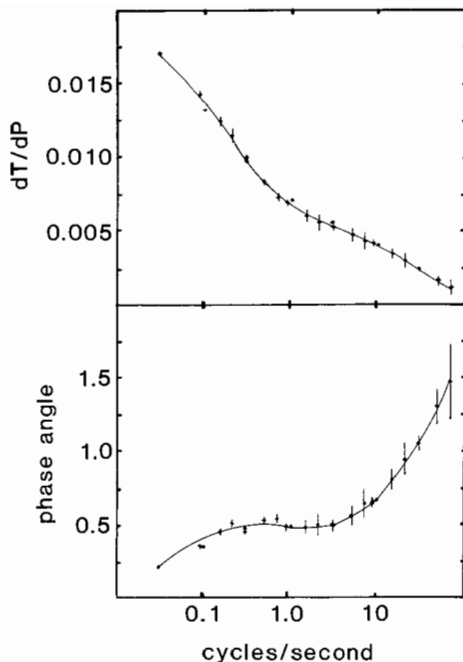


FIGURE 6 Fourier analysis of data obtained with the same DMPC solution described in Fig. 4 at 24.05 °C. The upper panel shows the amplitude of the pressure-induced temperature change as a function of the driving frequency. The lower panel is the phase angle of the driven temperature oscillation. Because this data has not been corrected for the dynamic response of the instrument, the amplitude decreases and the phase angle increases as the driving frequency approaches the response time of the thermistor ( $\sim 0.003$  s).

where  $Amp_i$  and  $\tau_i$  are the characteristic maximal amplitudes and the response time for each of  $i$  processes,  $\omega$  is the driving frequency of the pressure change and  $\Psi$  is the phase angle of the resultant temperature change.<sup>† 4,5,9</sup> We currently feel that the Fourier technique is the best method to analyze the experimental data in a model-independent fashion.

<sup>†</sup>The phase angle of the temperature change refers to the number of radians which the periodic temperature change lags behind the driven periodic pressure change. If the chemical reactions and the instrument response are rapid compared to the frequency of the driven pressure change, the phase angle would be zero. As these become significant compared with the driving frequency, the phase angle increases and the amplitude of the temperature oscillation decreases.

Figure 6 presents a Fourier analysis of the same solution of DMPC as in Fig. 4 at 24.05 °C. The upper panel is the amplitude of the driven oscillation ( $dT/dP$ ; °C/atm) and the lower panel is the phase angle of the driven temperature oscillation. Because this data has not been corrected for the dynamic response of the instrument, the amplitude decreases and the phase angle increases as the driving frequency approaches the  $\sim 3$  ms response time of the thermistor. The data also shows a very slow component with a characteristic response time of  $\sim 8$  s.

The corrections for the dynamic response of the instrument are easy to perform in the frequency domain. The effective gain and phase shift of the instrument are calibrated as a function of frequency with a sample which has fast relaxation times as compared with the time response of the instrument. For this purpose, we have used both distilled water and phospholipids 10 °C above the phase transition with similar results. The analogous corrections are extremely difficult to perform in the time domain.

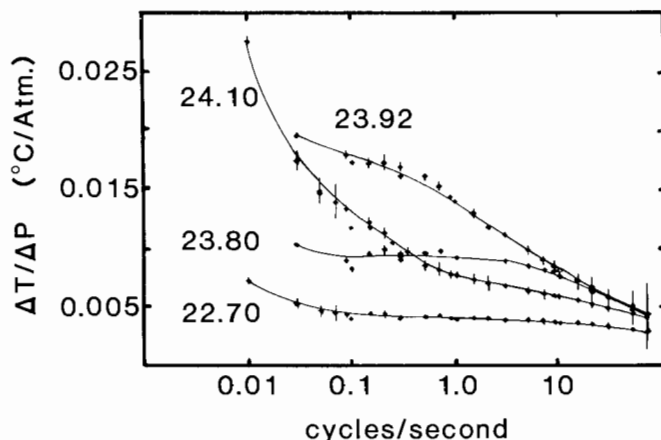


FIGURE 7 Same data as presented in Fig. 4 showing amplitude of the pressure-induced temperature change as a function of driving frequency at various temperatures. This data has been corrected for instrument dynamic response. The data at 22.7 °C is out of the phase transition region and shows a low amplitude, due to Joule-Thompson heating, and no phase angle. At the low temperature edge of the phase transition, 23.8 °C, the kinetics are rapid. As we traverse the phase transition, the kinetics become progressively slower while the amplitude increases. Table I shows an analysis of the data at 24.1 °C which indicates that this transition is a combination of at least four relaxation times.

Figures 7 and 8 show the same type of data as a function of mean temperature corrected for instrument response. The volume perturbation is only sufficient to force the lipids 20–30% of the way through the phase transition, so the data at successive temperatures represents only the kinetics in that limited region of the phase transition. The data at 22.7 °C is out of the phase transition region and shows a low amplitude, due to Joule–Thompson heating, and no phase angle. At the low temperature edge of the phase transition, 23.8 °C, the kinetics are rapid. However, as we traverse the phase transition, the kinetics become progressively slower while the amplitude increases. An analysis of this data at 24.1 °C indicates that it is a composite of at least four characteristic relaxation times. These are shown in Table I along with the corresponding

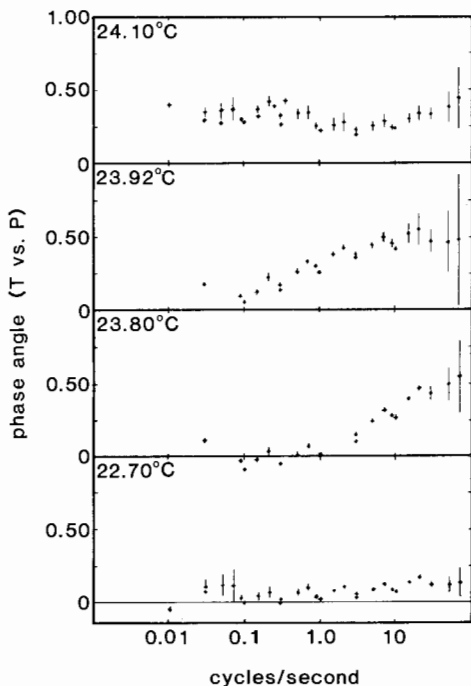


FIGURE 8 Same data as presented in Fig. 7 showing phase angle versus driving frequency at temperatures below the phase transition (22.7 °C) and at varying positions within the gel to liquid-crystalline phase transition.

TABLE I  
Apparent relaxation time constants of DMPC at 24.1 °C

$\tau_i$ (s)	% Amplitude
11.1 (6.7, 17.6)*	63.2 (45.1, 88.2)
0.69 (0.46, 0.91)	12.1 (10.2, 14.4)
0.22 (0.014, 0.031)	8.2 (5.2, 11.2)
<0.003†	16.3 (13.0, 19.6)

\*Confidence interval corresponding to  $\pm$ SEM.

†Faster than instrument response.

relative amplitudes and one standard deviation confidence intervals. The values of the time constants and amplitudes are highly temperature dependent within the phase transition.

From these limited data, a number of interesting conclusions can be drawn. If the gel to liquid-crystalline transition of multilamellar DMPC vesicles were a simple two-state transition, then the results in Figs. 7 and 8 and Table I would be characterized by a single relaxation time. Thus, the transition must be at least a two-step, three-state process. If we assume a two-step process, we can show that the characteristic melting temperatures ( $t_m$ ) of these processes differ by approximately 0.1 °C (see Fig. 9). The lower temperature transition, the dashed line in Fig. 9, is rapid, 20 ms or less, while the higher temperature transition, the dotted line, is approximately 10 s. The volume change of the phase transitions seems to be associated with the slow relaxation process (see the lower panel in Fig. 4).

## DISCUSSION

Light scattering, Raman spectroscopy, and fluorescence changes of marker dyes have been employed in studying phospholipid phase transitions. However, it is difficult to interpret changes in these observables in terms of thermodynamic state variables. The direct monitoring of the temperature and pressure obviates these problems. A side benefit of using the solution temperature as a monitor of the time course of the approach to equilibrium is an increase in the signal-to-noise ratio, which for this instrument is dependent on the number of cycles averaged. A typical value for the tem-

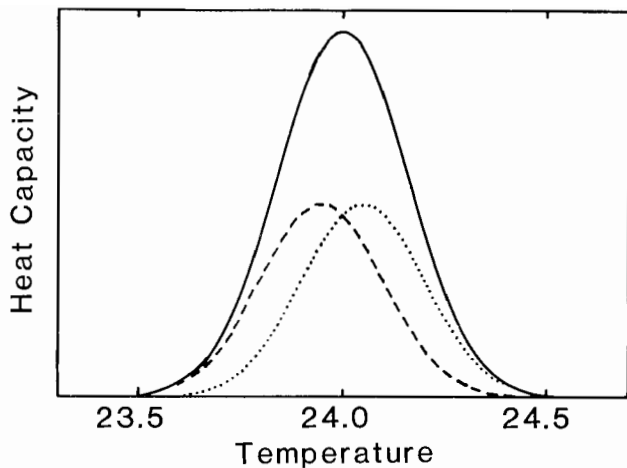


FIGURE 9 Depiction of a simulated three-state, two-step phase transition showing individual transitions, dashed and dotted curves, and the observable phase transition, the solid curve. An analysis of the data presented in Figs. 7 and 8 by a model of this type indicates that the characteristic melting temperatures ( $t_m$ ) of these two individual transitions differ by approximately 0.1 °C. The lower temperature transition, the dashed line, is rapid, 20 ms or less, while the higher temperature transition, the dotted line, is approximately 10 s. The volume change of the phase transition seems to be associated with the slow relaxation process (see the lower panel in Fig. 4).

perature uncertainty is  $\pm 2 \times 10^{-5}$  °C, which corresponds to a signal-to-noise ratio of more than 2000 in Fig. 4. When similar experiments were performed using light scattering<sup>10</sup> or Raman spectroscopy,<sup>8</sup> the signal-to-noise ratio was notably worse than those obtained with this instrument.

The analysis of the experimental data from this experiment can be performed by several methods. The data can be least-squares fit to multiple exponential functions to determine the characteristic chemical relaxation times directly. This data can also be used to directly test various molecular mechanisms by least-squares fitting the data to the integral of a system of differential equations which describes the particular molecular mechanism and the response of the instrument. This data also allows the calculation of the amplitude of the temperature and pressure change as a function of the frequency of the volume perturbations (e.g.,  $\Delta T$  versus  $\omega$  and  $\Delta P$  versus  $\omega$ ) by the use of Fourier transforms. This flexibility

allows us to measure the frequency and temperature dependence of the enthalpy and heat capacity changes for phospholipid phase transitions. A knowledge of these and their frequency dependence should greatly add to our understanding of the molecular interactions involved in membrane structure and function.

Many previous studies indicate that the phase state of a biological membrane is a determining factor in a number of biological processes vital to cells. It is clear that in a living organism temperature usually does not act as the trigger for the main phase transition. Natural membranes are usually fluid to well below room temperature. It is currently believed that changes in pH and the concentrations of divalent cations (particularly  $\text{Ca}^{++}$ ) are capable of initiating phase transitions at a local level in regions composed of phospholipids with head groups bearing net negative charge. These transitions include the main phase transition, and lateral phase separations.

The ion fluxes across membranes constitute the electrical activity of the nervous system and are controlled by membrane proteins which act as ion channels or gates. One might consider the general question of how these channels might be affected by their local lipid environment. For example, it is thought that the conformational state of a channel determines whether it is open, closed, or in some other state relative to ion passage. If channels are found preferentially associated with a particular phospholipid, such as phosphatidylserine, which is primarily negatively charged at physiological pH, then transmembrane  $\text{Ca}^{++}$  fluxes occurring during excitation might be expected to result in uptake of  $\text{Ca}^{++}$  by serine head groups. If sufficient  $\text{Ca}^{++}$  were bound by a domain of phosphatidylserine surrounding a channel protein, the lipid in this domain could be driven from a fluid to a gel state. Thus, greater lateral pressure would be exerted on the surrounded protein, perhaps changing the status of the channel through conformational changes in the protein itself. The time scale over which such changes might occur would be a function of the rate constants of the individual processes involved, including the transition of a phosphatidylserine domain from liquid-crystalline to gel phase (and the reverse), or phase separation of phosphatidylserine from phosphatidylcholine (as an example). Some of the time constants we have been able to resolve are sufficiently short that they could be

involved in the activity of the heart, where the action potential has a duration on the order of 200 ms. It is interesting to note that anesthetics such as benzocaine, procaine, tetracaine, dibucaine and lidocaine have been shown to have very pronounced effects on the equilibrium properties of artificial membranes, and in particular the cooperative unit size, i.e., the size of the domains formed during the phase transition.<sup>11</sup> It is also interesting that these same agents have antiarrhythmic properties on the heart. Little information is currently available about the effects which these anesthetics, and/or antiarrhythmics, have on the dynamics of membrane structure.

Rhodopsin is a second example of a biologically significant receptor system with properties which are directly related to membrane structure. Rhodopsin is an intrinsic membrane protein found in rod outer segment disk membranes. Upon absorption of a photon a series of spectrally defined intermediate forms of rhodopsin are formed sequentially. Bleaching of rhodopsin is believed to cause structural changes in the protein which then allows interactions with, and activation of, rod outer segment enzymes. These coupled reactions are believed to be involved in visual transduction with rhodopsin initiating the process by absorption of a photon. As rhodopsin goes through this cycle of events, it remains localized in the disk membrane. The rate of interconversion among certain spectral states of rhodopsin has been shown to be related to the physical and structural properties of its hydrophobic environment. The properties of the disk membrane may therefore be critically linked to the process of visual transduction.

In the section devoted to the description of our volume-perturbation calorimeter, it is shown that we have the capability to measure the frequency dependence of expansivity and the adiabatic compressibility of a phospholipid dispersion as a function of temperature. From these two response functions, the isothermal compressibility can also be calculated as a function of frequency and temperature. This isothermal compressibility,  $K_T$ , is a measure of the relative mean square volume fluctuations of the system. In studies of the response of lipid bilayers to ultrasonic radiation,<sup>12</sup> it was observed that  $K_T$  undergoes a small but significant enhancement in the temperature region of the main phase transition, on a nanosecond relaxation time scale. Such rapid decay of volume

fluctuations likely precludes them from importance in considerations of the mechanism by which large molecules such as proteins can be inserted in a model or natural membrane. However, large volume fluctuations, decaying on the order of 100 ms or slower (the time scales of the  $dT/dP$  and  $dP/dV$  relaxations which we have measured) are much more likely to be involved in the solvation of protein molecules by, or the passage of large molecules through, the bilayer.

In natural membranes, the gel to liquid-crystalline phase transition is very broad, implying that over a wide temperature range the volume fluctuations may be enhanced. Changes in concentration of divalent cations could cause the phase separation and freezing of phospholipids bearing net negative charge even though natural membranes usually exist above the temperature range of their main phase transition. By this mechanism portions of the membrane could be rendered a mosaic of regions in differing phases, analogous to the state of the membrane in the temperature induced main phase transition. It is thus a reasonable assumption that these large-scale volume fluctuations may be produced, and membrane permeability and penetrability altered.

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