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THE KINETICS OF THE MAIN PHASE TRANSITION OF AQUEOUS DISPERSIONS OF PHOSPHOLIPIDS INDUCED BY PRESSURE JUMP AND MONITORED BY RAMAN SPECTROSCOPY

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The sensitivity of the melting transition temperature of aqueous dispersions of dipalmitoyl- and distearoylphosphatidylcholine to hydrostatic pressure is used to allow measurement of the rates of isothermal freezing and melting of the lipids by rapidly changing the pressure. The degree of order of the lipids is measured by monitoring a ratio of two points in the Raman spectrum of the lipids which changes sharply at the melting temperature. Use of this Raman order ratio allows correlation between the order of the sample and the rates of transition in a manner which is impossible by monitoring only turbidity. Our longest relaxation times range upwards from a few seconds for both compounds. The freezing rates are slowest when the samples are initially fully melted, and the melting rates are slowest when the samples are initially frozen. These results imply that nucleation of the growing phase dominates the kinetics of both freezing and melting.

Introduction

Comprehensive understanding of the molecular structure of a multi-phase system must derive from study of both equilibrium behavior and the kinetics of the phase transitions. With the recent increase in interest in the structure and function of biological membranes a great deal of theoretical and experimental research has been performed on the equilibrium properties of phospholipid bilayers. Much is now known about the various transitions experienced by artificial lipid systems, particularly about aqueous suspensions of diacylphosphatidylcholines. As lipid phase transitions have been implicated in control of function in biological membranes, there is a clear biological interest in the rates at which such phase transitions could taken place. Yet very little is known of the rates of phase transitions in phospholipid systems, let alone the factors which control those rates. For the pretransition in dipalmitoylphosphatidylcholine, two sets of rates have been published which differ by several orders of magnitude [1,2]. Few kinetic studies have been made of the faster main transition.

While the theory of rates of phase transitions is by no means a new topic [3], there exists no well-established theoretical method for analyzing transition kinetics in phospholipid systems. One reason for this is that the aqueous dispersion (the most commonly studied system) is made up of discrete liposomes, each of which contains discrete bilayers made up of numerous molecules which are themselves polymers. Despite this discouraging degree of organizational complexity, fruitful attempts have been made to fit the transition kinetics with simple models.

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One detailed kinetic study of the main transition of phosphatidylcholines [1] involved applying sucessive small temperature jumps and measuring rates of melting at various temperatures through the transition temperature range. The authors interpreted their results to be in support of a simple kinetic Ising model similar to that employed in the study of linear polymers [4]. However, they used the turbidity of the dispersion to monitor the state of their samples, a method subject to irreversible effects and one by which only relative degrees of order can be obtained from individual measurements. Sample heterogeneity which can have unpredictable effects on turbidity of lipids [5] was not expressly addressed as it has been in other studies [6].

Another more recent study utilized a pressure-jump technique to induce rapid melting, but also monitored relaxations by sample turbidity [7]. Between this and the previous study there is good qualitative but poor quantitative agreement on the melting relaxation times. Both concur that relaxation lifetimes reach a maximum at the center of the main phase transition for vesicles or dispersions, but disagree on the interpretation of the phenomenon.

Our approach, briefly presented elsewhere [8], has been to use Raman spectroscopy of the phospholipid hydrocarbon chains to monitor the state of the lipids. Raman scattering can be used to generate parameters which can be interpreted as an absolute degreee of order, consistent from sample to sample. Our 'order parameter', based as it is on a ratio of two Raman bands, should not be affected by changes in refractive index, size of liposomes or their degree of aggregation, thereby freeing the results from confusion with relaxations with no basis in the structure of the bilayer itself.

We have utilized the dependence of the melting temperature (T_m) on the applied hydrostatic pressure [9] to perturb the sample with a rapid change in pressure. This dependence of the transition temperature is due to the change in volume which occurs upon melting (ΔV_t), measured directly by dilatometry with an oberved value of 0.037 ml/g for dipalmitoylphosphatidylcholine [10,11].

This value of ΔV_t has also been estimated from the Clausius equation applied to the dependence of the transition temperature on applied pressure [12]. Because of the apparent linear increase in the melting temperature with applied pressure and the constancy of the width of the transition [11] at the elevated pressures, we believe that one can use the change in pressure and the change in temperature as interchangeable methods by which to perturb the equilibrium of the sample, as we have shown in our previous work on pressure-induced phase transitions on this system [13]. In other words, the microscopic mechanism by which a dispersion sample would respond to a perturbation in equilibrium should be independent of whether the perturbation were brought about by pressure or temperature. One should, therefore, be able to induce rapid isothermal phase transitions. The recent work of Grunewald et al. [7] confirms the merit of the technique.

The greatest advantage of using a pressure-jump technique over a temperature jump is that with the pressure jump one can rapidly freeze a sample as well as melt it. We present here the first set of kinetic data on freezing of phospholipids. While we could not produce a pressure-jump as fast as the microsecond time scale available with commercial temperature-jump devices, slower pressure changes have proven adequate for our samples. Nuclear magnetic resonance measurements on lipid dispersions at T_m show two distinct types of lipid present on a millisecond time scale (Dahlquist, F.W., personal communication), and the work of Tsong and Kanehisa [1] indicated the presence of a broad range of relaxation times ranging from milliseconds to seconds. Our apparatus is certainly capable of following the slower relaxations, and the amplitudes of the very fast relaxations have proven to be so low as to be unimportant in this study.

Materials and Methods

The L-isomers of 1,2-dipalmitoylphosphatidylcholine and 1,2-distearoylphosphatidylcholine were purchased from Sigma Chemical Co., and were further purified by column chromatography on Sephadex LH-20 in 95% ethanol at 40°C. All samples showed one spot by thin-layer chromatography. The pooled tubes from the chromatographic runs were dried in a rotary evaporator, leaving a glass-like film on the interior surface of

the flask. This was then placed under high vacuum for at least 12 h to remove traces of ethanol. Aqueous dispersions were formed by addition of glass-distilled water to the flask and gently swirling the contents while immersed in a bath at temperature above T_m.

The high-pressure Raman cell used in these experiments, as well as the Raman order ratio used to quantify the order in the samples, have been described elsewhere [14]. The apparatus for delivering rapid changes in hydrostatic pressure is shown in Fig. 1. A tank of helium at approximately 14 MPa (140 atm) is attached to a high pressure regulator (Victor) which is used to bring the pressure down to the upper pressure used in the experiments. Tubing in the system between the solenoid valves (Skinner Valve) and the sample is filled with distilled and degassed water. The 'tee' fitting between the solenoid valves SV1 and SV2 eliminates vacuum formation at the sample during gas flows, and in combination with the water filled tubing allows rapid pressure re-equilibration. No evidence of dissolution of helium in the sample was seen in the experimental periods, thanks to the water in the tubing and the small fluidizing effect of the gas on lipids [15,16].

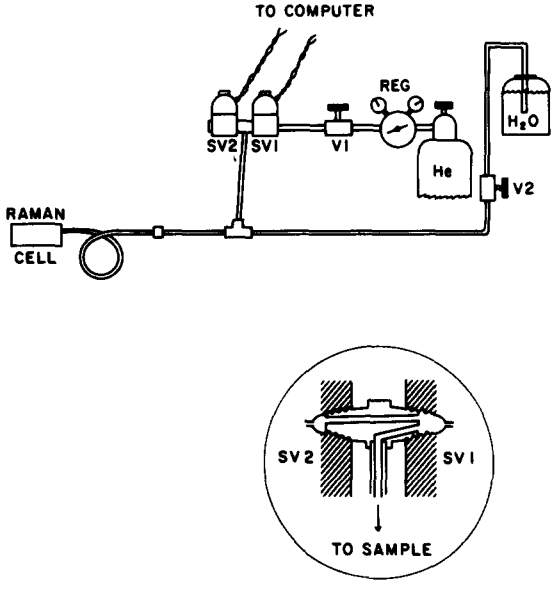

Fig. 1. A schematic diagram of the hydraulic and gas flow apparatus portions of the pressure-jump system. The inset is a detail of the 'tee' fitting between the solenoid valves. V1 and V2 are valves, SV1 and SV2 are solenoid valves, and the Raman cell is shown in detail elsewhere [14].

Details of the Raman scattering experimental setup and computer-controlled operations have been published elsewhere [13,14]. Spectra were collected with 200 mW of 514.5 nm laser excitation, with the SPEX 1301 monochromator's slits set at 250 μm. The melting curves shown here were generated by the same procedures as described in the above-mentioned publication from this laboratory. Many Raman spectroscopic studies of lipids have quantified the order of the samples by measuring the ratio of the intensities of the two highest points of the C-H stretching manifold. However, the width and position of these bands change near the transition temperature [17], which ruled out the usual type of order measurement in the absence of multichannel recording capabilities. The Raman order ratio is defined by the following:

$$\text{Raman order ratio} = \frac{I_{(2880)} - I_{(2790)}}{I_{(2847.5)} - I_{(2790)}} \qquad [1]$$

It is not strictly comparable to peak height measurements made in other laboratories. For example, the peak height ratio of the asymmetric to symmetric stretching bands of dipalmitoylphosphatidylcholine dispersions was observed by one laboratory to reach a limiting value of about 2.0 at -40°C [18]. The Raman order ratio reached 1.6 at the same temperature after equilibration for 0.5 h.

For 'equilibrium' melting and freezing curves at a constant hydrostatic pressure, the temperature was scanned at an overall rate of 0.85 K/h, taken in discontinuous steps of 0.1 K for dipalmitoylphosphatidylcholine. The temperatures discussed in this paper are those of the circulating bath, since the temperature of the sample in the capillary at the laser beam focus cannot be determined directly. The relaxation of the sample to rapid changes in pressure was obtained by repetitively recording the scattered light intensity before and just after the pressure-jumps at the three Δcm^{-1} shifts of the Raman order ratio. A total of six relaxation curves of 1001 points each were generated for the pressure increase and decrease with 50 ms collection time per point. A 55 s delay was inserted after each relaxation in order to as-

sure that the sample had come to equilibrium. If the relaxation rates observed are sufficiently fast, the first 251 data on each curve can be used as the t_0 and t_∞ points for subsequent fitting procedures.

Processing of the kinetic data was performed in FORTRAN on a Varian V76 computer. For each temperature, the six relaxation curves were processed according to Formula 1 in order to produce a melting curve and a freezing curve. These were fit to both a single exponential relaxation equation and the sum of two exponentials between the endpoint values. The fitting program [14] followed a gradient-search least-squares approach [19] and was modified for use on this data from a program previously developed in our laboratory [20]. The fits were optimized by minimizing the sum of the squared deviations. The magnitude of the pressure-jump was kept constant and we varied the temperature at which the sample was maintained. The choice of 3.45 MPa as the pressure-jump was a compromise between maximizing the total change in Raman order ratio to increase the signal-to-noise ratio and minimizing the range of order covered to obtain kinetic data which approximated as well as possible the rates for infinitesimal changes in order. The final signal-to-noise ratios of the kinetic curves varied widely from experiment to experiment due to varying total changes in the Raman order ratio between the two pressures, or to differences in the total collection time.

Results

Most of our experiments were performed on dispersions of dipalmitoylphosphatidylcholine, and most of the data presented here were generated from a single dispersion sample with this phospholipid. In addition, we present data from a sample of distearoylphosphatidylcholine for comparison, and in Fig. 8 we have pooled kinetic data from two previous dipalmitoylphosphatidylcholine experiments to indicate trends in the relaxation rates more clearly.

The purity of the lipid samples is indicated by the presence of a pretransition and the narrowness of the main melting transition. For dipalmitoylphosphatidylcholine the width of the melting transition can be seen in Fig. 2 to be about

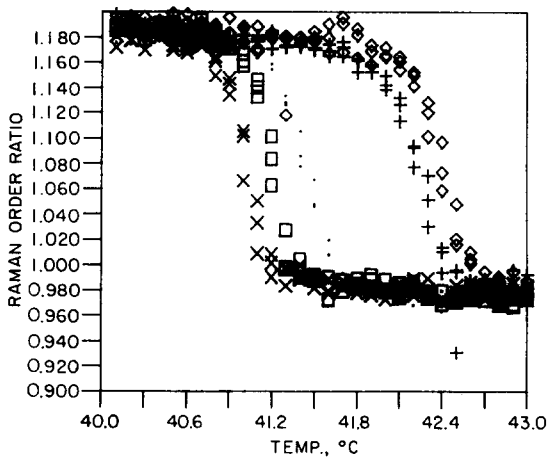

Fig. 2. The melting and freezing curves of a dipalmitoylphosphatidylcholine dispersione at atmospheric pressure, and at 3.45 MPa. Note that the chain melting transition shown here occurs between Raman order ratio values of approx. 0.97 and 1.19, (□, melt at atmospheric pressure; ×, freeze at atmospheric pressure; ◇, melt at 3.45 MPa; +, freeze at 3.45 MPa; ·, melt at atmospheric pressure taken at end of experimental sequence).

0.3 K from the beginning to the end of the transition as monitored at atmospheric pressure. This is only about twice the width of the best published calorimetric phase transition curve on ultra-pure dipalmitoylphosphatidylcholine [21], and is substantially narrower than many published transition curves for 'pure' dipalmitoylphosphatidylcholine. A similarly narrow transition width and pronounced pretransition were observed for distearoylphosphatidylcholine, with the exception that the center of the main transition was observed at higher temperature and that the 'edges' of the transition are at Raman order ratio values of 0.97 and 1.13. There is, despite an extremely slow scan rate, a slight offset of the melting and freezing curves of all samples as exemplified by the data on dipalmitoylphosphatidylcholine seen in Fig. 2. It is probable that the apparent hysteresis is an artifact of the cell design, possibly aggravated by the manner in which the equilibrium melting curves were recorded (changing the temperature, then scanning to the three Δcm^{-1} shifts in sequence). The best equilibrium values for the order parameters are probably obtained by averaging the Ra-

man order ratios obtained from the freezing and melting curves.

Two curves in Fig. 2 which were obtained at 3.45 MPa were taken at the end of the series of pressure jump experiments, nearly 5 days after the atmospheric pressure curves. They still show a narrow transition with some hysteresis, but shifted to higher temperatures as one would expect. All curves become essentially superimposable at temperatures below 40.0°C and above 44.0°C. A fifth curve, which is an atmospheric pressure melting curve taken after the 3.45 MPa melting and freezing curves, is shifted by about +0.25 K from the melting curve taken several days earlier at atmospheric pressure, and is also slightly broader. This should not seriously affect the kinetic measurements reported here; this change is probably not caused by degradation, as degradation of samples usually tends to result in lowering of their melting points.

Fig. 2 gives us an opportunity to compare the shift of the dipalmitoylphosphatidylcholine melting curve with that predicted by more accurate measurements of other laboratories. Using 0.0237 K/atm as a average value of dT_m/dp [9], 3.45 MPa (34 atm) should shift the melting transition by 0.81 K. This is approximately the shift in temperature seen between the 3.45 MPa melting curve and the final atmospheric pressure melting curve in Fig. 2, and substantially less than 1.2 K shift seen between the first and second sets of curves. Some alteration in the properties of the sample or of the system as a whole must have taken place during the five day course of the experiment. Consequently, we cannot use equilibrium values such as those given in Fig. 2 to give t_0 and t_∞ values for the kinetic experiments as the temperatures indicated may be off by as much as 0.2 K. The internal consistency of our data reassures us that the changes during the course of the individual kinetic curves was slight and the qualitative agreement between the results on the two sets of lipids indicates that any drift in sample conditions would not affect the conclusions derived from the kinetic data.

Fig. 3 is a plot of the Raman order ratio versus applied pressure for dipalmitoylphosphatidylcholine at 43.0°C. It takes about 6 MPa to bring the

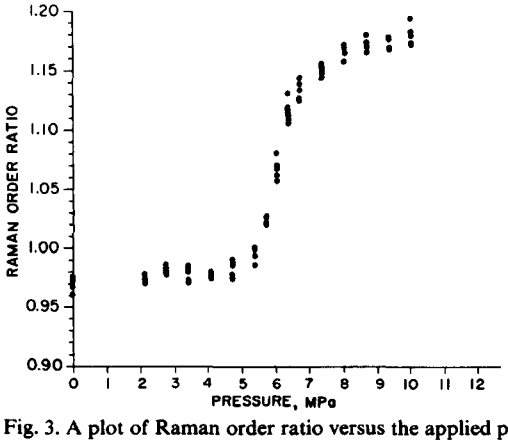

Fig. 3. A plot of Raman order ratio versus the applied pressure, with the dipalmitoylphosphatidylcholine sample held at 43.0°C. All points were taken in the direction of increasing pressure, with 5 to 7 points shown at each gauge setting and averaging times of 100 s per point.

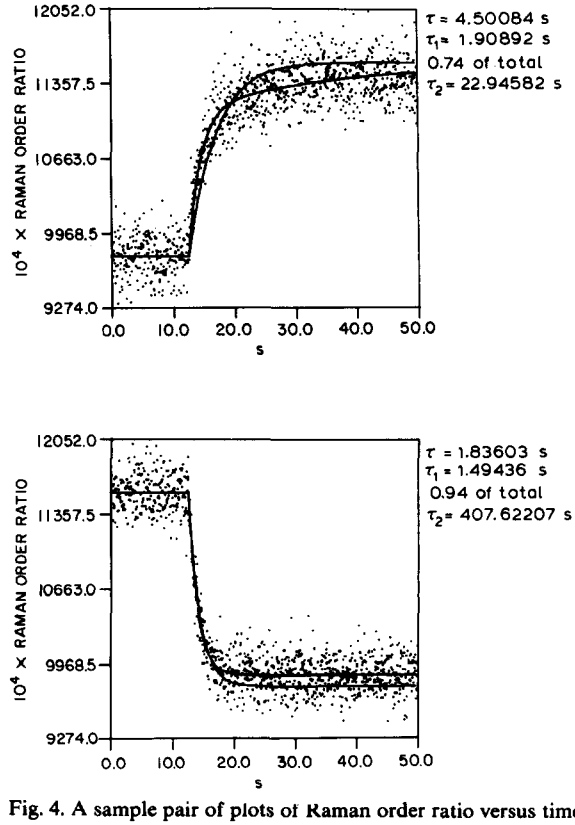

Fig. 4. A sample pair of plots of Raman order ratio versus time for dipalmitoylphosphatidylcholine with the best fits for single exponential decays and the sum of two exponentials superimposed. This set was generated with the sample at 41.9°C and demonstrates a signal-to-noise ratio which is 'typical'.

Raman order ratio to its midpoint value of 1.07 from the starting value of 0.97. This pressure should shift the T_m by 1.4 K, which is in near agreement with the Raman order ratio 1.07 point on the final melting curve at atmospheric pressure.

An example of raw kinetic data is shown in Fig. 4. In this case the sample was dipalmitoylphosphatidylcholine at 41.9°C. The signal-to-noise ratio was often adequate to resolve two exponentials in the relaxations, but in many cases, particularly for melting curves, a single exponential was clearly sufficient. The data shown in Fig. 4 is for one of the larger jumps in Raman order ratio, but the worst sets of curves have signal-to-noise ratios which are poorer by no more than a factor of three. Reproducible fits to the data would be obtained within a few iterations of the computer program. The full set of curves as well as tabulated relaxation rates for the dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine samples may be found elsewhere [14]. The values of the Raman order ratio at the calculated t_0 and t_∞ for both the dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine samples are plotted in Fig. 5. If the t_0 and t_∞ points calculated from the kinetic data corresponded well with the low and high pressure 'equilibrium' melting curves as shown for dipalmitoylphosphatidylcholine in Fig. 2, we could safely say that the 105 s between changes in the pressure were sufficient for the sample to have come to equilibrium. This is quite important as the curve fitting program uses the 251 points at the start of each data set as 'true' end points. It appears that equilibrium Raman order ratios were not reached for relaxations to high pressure at high temperature, or for relaxations to low pressure at low temperatures. This means that for these transitions the relaxation lifetimes must have components on the same order of magnitude as the pressure cycling time or longer.

Improvement in the sum of the deviations squared for individual fits indicated that two or more exponentials usually fit the relaxation curves better than a single exponential, particularly in the freezing direction. However, the single exponential lifetimes are useful to indicate general trends in the data. In this light we have plotted the lifetimes for freezing and melting versus temperature in Fig. 6. From these data the trends are clear. At low temperatures, where the samples are more ordered, the lifetimes for melting are longest, while at high temperatures, where the samples are more disordered, the lifetimes for freezing are largest. The range of rates is quite large, being over two orders of magnitude for the change in the dipalmitoylphosphatidylcholine freezing rate.

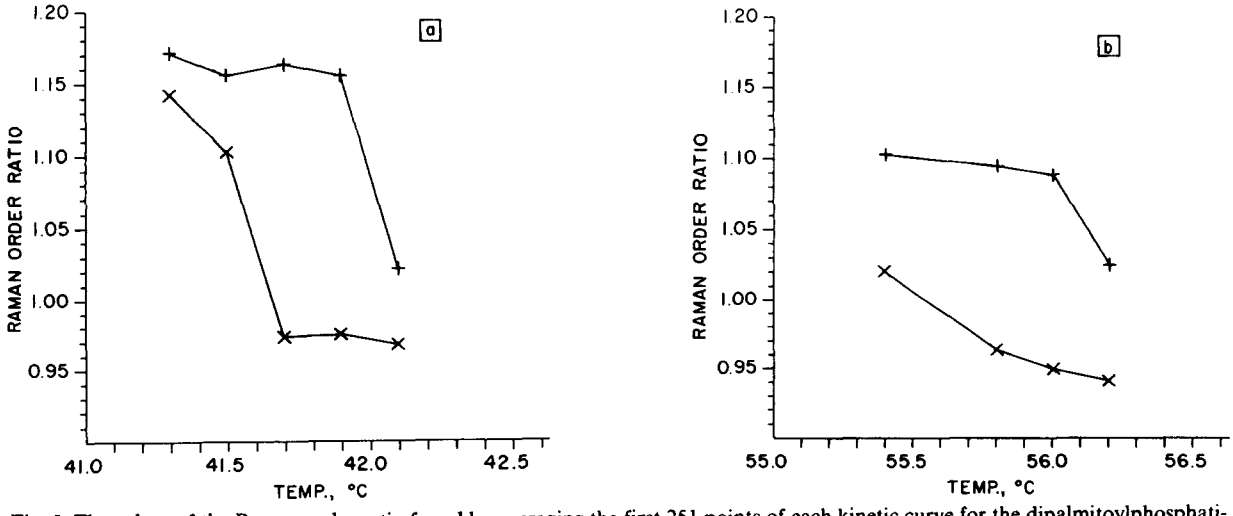

Fig. 5. The values of the Raman order ratio found by averaging the first 251 points of each kinetic curve for the dipalmitoylphosphatidylcholine (a) and distearoylphosphatidylcholine (b) samples plotted versus the temperature at which the measurements were made. (+, high pressure; ×, low pressure).

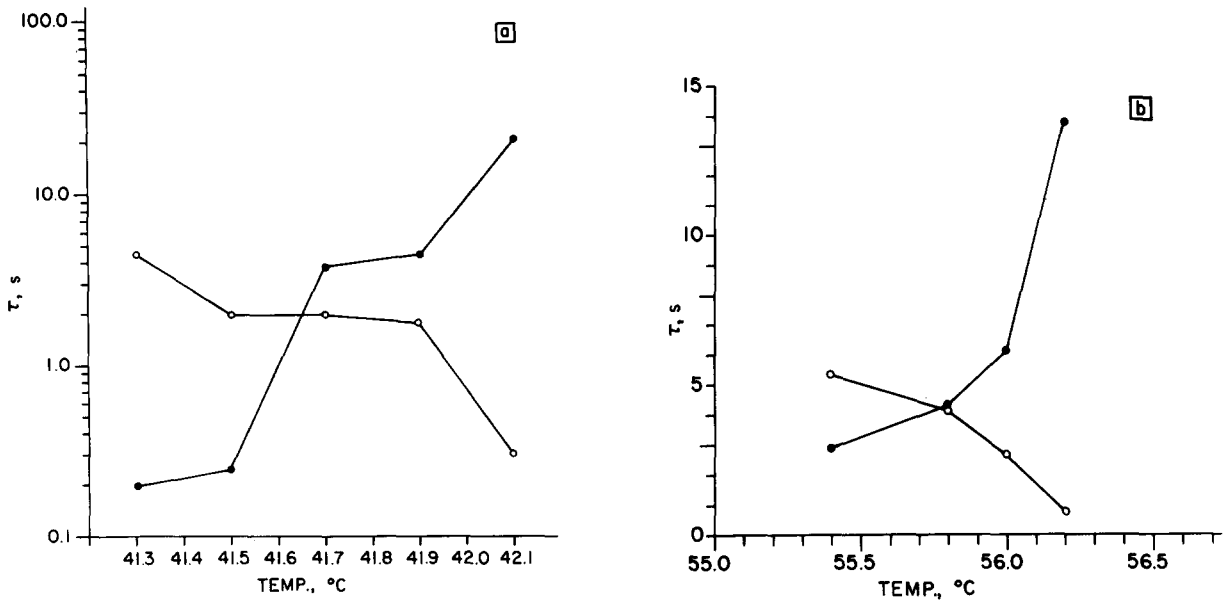

Fig. 6. Plots of τ, the single exponential lifetime in seconds versus the bath temperature of experiments with dipalmitoylphosphatidycholine (a) and distearoylphosphatidylcholine (b). (●, freezing lifetimes; ○, melting lifetimes).

Having established an order parameter of sorts which should change linearly with the proportion of lipid molecules in the solid and liquid states and subsequently having monitored relaxations of this Raman order ratio at various temperatures, we have the ability to relate the relaxation rates to the state of the lipids in a way which is impossible when only the experimental temperature is known.

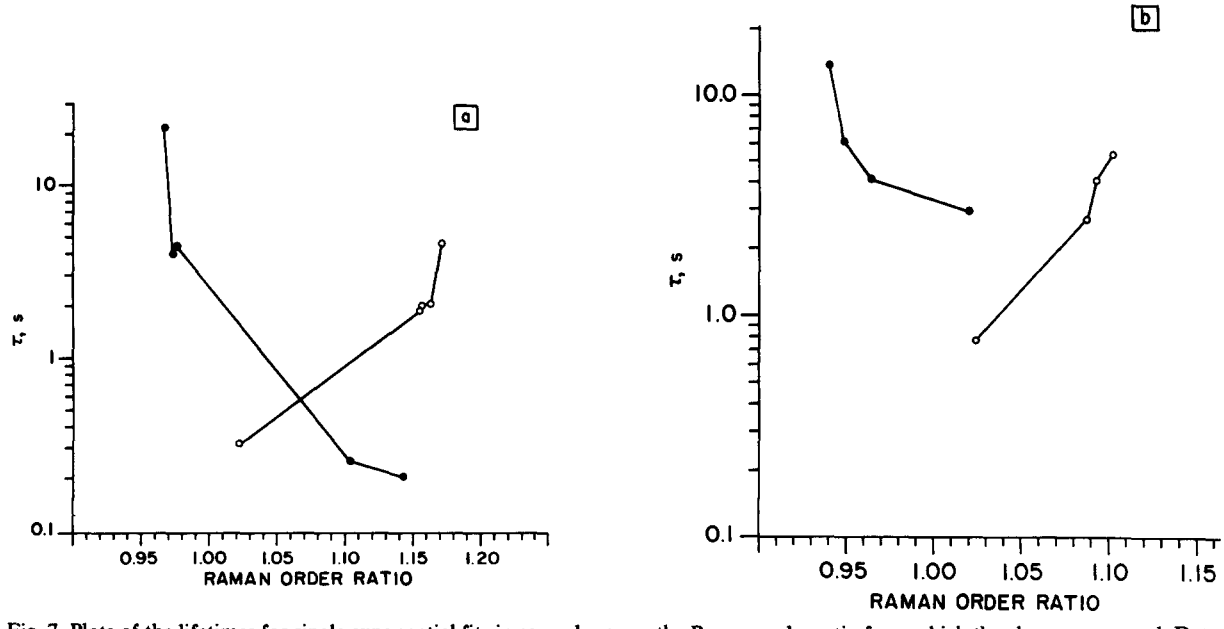

Fig. 7. Plots of the lifetimes for single exponential fits in seconds versus the Raman order ratio from which the changes occurred. Data are shown for dipalmitoylphosphatidylcholine (a), and for distearoylphosphatidylcholine (b). (●, freezing lifetimes; ○, melting lifetimes).

We have observed no correlation between the change in Raman order ratio induced by the pressure jump and the relaxation times. This rules out a number of possible interpretations of the data, as will be discussed below.

It is also possible to plot the relaxation times as a function of either the initial or final order observed for those relaxations. In Fig. 7 the melting and freezing lifetimes are plotted versus the Raman order ratio at which the melting or freezing processes begin. It appears that melting would continue to slow at Raman order ratios greater than those in the transition region and that freezing would continue to slow at Raman order ratios below the transition. When one measures rates in the extreme regions of the transition and beyond, it is found that the amplitudes of the slow processes tend to zero as their lifetimes increase. Outside the range of Raman order ratios of about 0.97 to 1.19 for dipalmitoylphosphatidylcholine, and a range of 0.97 and 1.13 for distearoylphosphatidylcholine, there is no freezing or melting as defined near the transition temperature, and one is observing rapid relaxations of a single-phase bilayer system. The trends of the relaxation lifetimes which have sufficient amplitude for us to observe indicate that both freezing and melting can be rate-limited by nucleation. Also note the close qualitative and quantitative agreement for the two lipids studied.

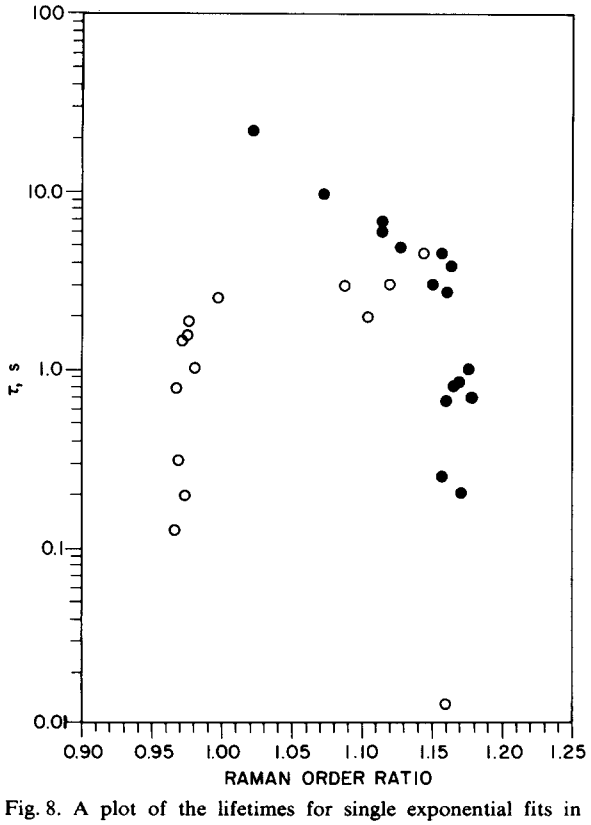

Fig. 8. A plot of the lifetimes for single exponential fits in seconds versus the Raman order ratio reached at the end of the relaxation. Data are pooled from two sets of experiments on dipalmitoylphosphatidylcholine in addition to the set shown in the rest of this paper. (●, freezing lifetimes; ○, melting lifetimes).

Alternately, one can analyze the relaxations in terms of the final Raman order ratio. For infinitesimal changes in order, this would be equivalent to comparing the relaxation time to the degree of transition, but for finite order changes such as ours it is not so clear cut. We have assembled all our data from dipalmitoylphosphatidylcholine in Fig. 8 and plotted it in this manner. The data on distearoylphosphatidylcholine followed identical trends. The relaxation times rise rapidly from the edges of the transition, at which the rates are extremely rapid, to a level of about 2 to 10 seconds through the majority of the phase transition (which is, again, centered near a Raman order ratio of 1.07). For melting, at least, the relaxation times are seen to decrease again at high Raman order ratios, but we have no data in the low Raman order ratio range where the freezing lifetimes might again decrease.

Discussion

Both our technique and results differ in certain significant aspects from those found in previously published work. We wish to outline possible systematic differences which may be responsible for some of the disagreement. For example, our liposome samples are dispersed in distilled water. Gruenewald et al. [7] hypothesized that relaxations in the second range and slower are an aggregation phenomenon only seen at the high salt concentrations required for temperature-jump experiments. We, of course, observe melting relaxations in this range and slower in the absence of salt. Our samples, however, have been centrifuged to the bottom of a capillary tube, resulting in contact between liposomes which may either resemble or promote a

state of aggregation. The extremely slow relaxations seen by us and others may reflect a restriction of the rate of the phase transition for aggregated liposomes, due probably to the requirement for cooperative phase transitions in the aggregated liposome system. The fact that there is a controversy concerning the presence of the slow relaxations highlights the importance of the large lipid structures in controlling the rates of the transitions. Under any circumstances the Raman order ratio would not be sensitive to a change in the aggregation state of the liposomes unless this aggregation directly or indirectly altered the order of the phospholipid molecules. This points out a major difference between use of turbidity and a molecular spectroscopic technique to monitor a phase change – all of our signal change is due to alterations in molecular structure.

Since our samples are prepared at much higher concentration than is used for turbidity measurement, there are several possibilities for mechanical artifacts which must be considered in analysis of our data. Both volume and latent heat changes in the sample must be considered as possible sources of rate-limiting processes. An anhydrous dipalmitoylphosphatidylcholine dispersion (impossible as it may be) would change volume by about 4% at the chain melting temperature [10]. Since our samples are at least 50% water by weight, the overall change in volume for our samples can be no more than 2%. If the order changes are rate-limited by the flow of water into and out of the sample cell to accommodate the volume change induced by swelling or contracting of the sample, the slowest rates should be observed simultaneously with the greatest change in the order regardless of the direction of the phase transition. This is not so for our data.

Other problems would occur if the rate limiting step were heat transfer to or from the sample due to the latent heat which accompanies any order change. The enthalpy of the main transition for dipalmitoylphosphatidylcholine is 8.74 kcal/mol [21], or 11.9 cal/g. If one were to imagine a 50% dispersion which suddenly jumped from one state to the other, there would be an instant change of temperature of nearly 6 K in the sample. Clearly, even for small jumps, heat flow into and out of the sample could be a possible rate-limiting step. We believe that since our sample is less than 50% lipid by weight, and the sample is contained in a tube with a bore of only 0.5 mm that the heat flow is sufficiently fast so that it is not the rate-limiting step. Also, the greatest heat flow and consequently the slowest rates again would be expected to accompany the maximal change in order. Our data does not support this.

Having eliminated concern that our pressure-jump apparatus or sample arrangement are creating artifactual relaxation rates, we wish to address the problem of the use of the Raman order ratio to measure the sample order. Both previous turbidity-measured kinetic studies [7,12] have attempted to correlate their observations with a reaction parameter θ which represents the fraction of molecules in the solid state. It is, of course, impossible to know from a single turbidity measurement what value of θ to apply to a sample; consequently, it is only by indirect and error-prone methods that one may determine between which two values of θ a particular relaxation in order occurred. A measurement of order based on molecular properties, however, should not be prone to any of these types of uncertainties. There is, in effect, a Raman spectrum for a 'solid' lipid molecule at a temperature just below the phase transition, and a different spectrum for a 'fluid' lipid molecule. Because of the rapidity of the change in order near T_m as compared to the surrounding temperatures it is possible to define values of the Raman order ratio which nearly correspond to the $\theta = 0$ and $\theta = 1$ points of the transition. The time scale of the Raman effect is so fast (10^{-13} s) that it is reasonable to assume four our purposes that all molecules in the laser beam will be either solid or fluid, and a negligible number will be in a spectroscopically (structurally) intermediate state. Within the transition region, therefore, the Raman order ratio should track the phase transition linearly. One could place a linear θ scale along the x-axes of Figs. 7 and 8 between the values of the Raman order ratio corresponding to the edges of the transition.

Our data does not necessarily fit any of the models which have been presented for the phase transition. The standard Ising model calculation for order-disorder transitions both for polymers as well as for lipids have predicted the slowest rates

at the midpoint of the phase transition. This was pointed out by Schwarz [4] who noted that according to the simple Ising statistics, the mean relaxation time shows a very sharp maximum at the midpoint of the order-disorder transition. He states that this is because at this point, for a given change in temperature, a maximum number of 'helix units' must be formed or destroyed. Despite its failings, the kinetic Ising model does provide a convenient framework within which our data may be interpreted. Unfortunately, we were forced to induce relatively large changes in order to be able to observe the relaxations. Most theoretical treatments of Ising models produce only the instantaneous relaxation rates. For finite changes in order some authors [7] have interpreted the model as indicating that the relaxations should be the slowest when the final order is at the midpoint of the transition. Our data from Fig. 8 indicate that the slowest relaxations are observed when the final Raman order ratio is anywhere within the phase transition, and there is no sharp peaking of the relaxation times. What we seem to be observing, rather, is a characteristic relaxation time in the range of 2 to 20 s within the center of the transition, with the freezing rates being slightly slower. This does not contradict the application of the kinetic Ising model to the interpretation of the data, but the data in and of themselves are insufficient to prove the validity of the model.

There is a somewhat simpler interpretation of our data, and we believe it successfully explains all published data on the kinetics of phase transitions in liposomes. That is that the rate determining step of the phase transition kinetics is the nucleation of the growing phase. In terms of the conceptual framework of the Ising model, our data, both with dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine as shown in Fig. 7 indicate that relaxation times are longest when the initial cluster size is smallest. (A cluster is always composed of the material present in the non-dominant amount). If the non-dominant phase is liquid, a pressure jump which converts liquid to solid is slow, and a pressure jump which converts solid to liquid is the most rapid.

We would like to point out Fig. 3 of the Tsong and Kanehisa paper [1] which shows the dependence of the cluster size on temperature. In almost every case both above and below the melting transition the dominant cluster size is zero. We take this to mean non-nucleated liposomes. Below the phase transition temperature, T_m, there are a large number of liposomes with no liquid clusters, while above the T_m there are a large number with no solid clusters. If we make the assumption that nucleation is by far the slowest step, the longest relaxation times will be dominated by the number of non-nucleated liposomes. The number of growth nuclei per multilamellar liposome is probably small. (In the model which we used previously to discuss the statistical mechanics of the pressure- and temperature-induced phase transitions we assumed only one nucleus per liposome) [13]. Growth may become progressively faster as the fraction of the growing component increases because of the decreasing number of liposomes which are unnucleated.

Strong dependence on the degree of nucleation is quite consistent with kinetics of melting of liposomes published elsewhere [1,7]. The fact that in our data the melting and freezing τ versus Raman order ratio plots are so symmetrical argues strongly that the rate limiting step is the same for the two processes. What is most striking from our data is that the relaxation for the phase changes are so slow. There are components of the relaxations, particularly for freezing, on timescales greater than tens of seconds and these have quite appreciable amplitudes. The differences between the melting rates observed by our technique and others may be caused by their dropping the slowest observed relaxations from their data analyses, or by the aforementioned problems of sample heterogeneity in turbidity measurements. We could have probably resolved some faster rates had we fit our data to the sum of three or more exponentials, as opposed to just two, but the data quality does not warrant such efforts. The wide range of relaxation rates we observe for most transitions (see Fig. 4) might represent separation of nucleation and growth rates, but are more likely due to the heterogeneity of our samples. A typical aqueous dispersion may contain multi- and unilamellar liposomes whose diameters vary over more than two orders of magnitude (Yager, P. and Peticolas, W.L., manuscript in preparation), with correspondingly enormous differences in the number of molecules per liposome.

Support for the dependence of the relaxation times on the initial order parameter may be derived from a consideration of the mechanical processes involved in converting a multilamellar liposome from one equilibrium state to the other. Large changes in area of the bilayer occur during the phase transition [22]. We have made a light microscopic study of the changes in shape of multilamellar liposomes (Yager, P. and Peticolas, W.L., manuscript in preparation) which has confirmed the assumption that interconversion between high- and low-temperature forms involves large changes in dimensions of liposomes.

The formation of a small non-dominant patch requires a mechanical distortion of the surrounding dominant phase both within the bilayer and in bilayers stacked above and below. The more embedded in surrounding bilayers is a potential nucleation site, the more difficult is it for the nucleus to form. Once the nucleus has been formed, growth many proceed at a more rapid rate which depends upon factors including the rate in which the overall shape of the liposome can be deformed, and the transfer of water across the bilayers. The initial nucleation within a liposome, though, is a highly cooperative process, and as such is a statistically unlikely occurrence. This is presumably partially responsible for the observed differences [1,7] in relaxation rates between small unilamellar vesicles and large multilamellar liposomes.

In conclusion, we find that our data, while not inconsistent with the predictions of a kinetic Ising model, or with the other published work, do not clearly support the model. We prefer to adopt the more general conclusion that the rate limiting step in the interconversion between solid and fluid states is nucleation of the subdominant phase in each liposome. The exponential lifetimes of the phase change may well by highly dependent on the conditions of sample preparation, but in our hands are on the order of 10 s for freezing and 2 to 5 s for melting.

We believe that this technique has great promise for studying the rates of other phase transitions. Pressure-jump, in conjunction with Raman spectroscopy, provides a uniquely informative method by which to study the time-dependent processes which occur as lipid systems relax to a new equilibrium state.

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