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PHASE TRANSITIONS IN PHOSPHOLIPID VESICLES

FLUORESCENCE POLARIZATION AND PERMEABILITY MEASUREMENTS CONCERNING THE EFFECT OF TEMPERATURE AND CHOLESTEROL

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SUMMARY

We have studied the solid to liquid–crystalline phase transition of sonicated vesicles of dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine. The transition was studied by both fluorescence polarization of perylene embedded in the vesicles, and by the efflux rate of trapped $^{22}Na^+$.

Fluorescence polarization generally decreases with temperature, showing an inflection in the region 32–42 °C with a mid-point of approximately 37.5 °C. On the other hand, the perylene fluorescence intensity increases abruptly in this region. To explain this result, we have proposed that, for $T < T_c$, where T_c is the transition temperature, perylene is excluded from the hydrocarbon interior of the membranes, whereas, for $T > T_c$ this probe may be accommodated in the membrane interior to a large extent.

The self-diffusion rates of $^{22}Na^+$ through dipalmitoylphosphatidylglycerol vesicles exhibit a complex dependence on temperature. There is an initial large increase in diffusion rates (approximately 100-fold) between 30 and 38 °C, followed by a decrease (approximately 4-fold) between 38 and 48 °C. A monotonic increase is then observed at temperatures higher than 48 °C. The local maximum of $^{22}Na^+$ self-diffusion rates at approximately 38 °C coincides with the mid-point of phase transition as detected by changes in fluorescence polarization of perylene with the same vesicles. Vesicles composed of dipalmitoylphosphatidylcholine show the same general behavior in terms of $^{22}Na^+$ self-diffusion rates at different temperatures, except that the local maximum occurs at approximately 42 °C.

The temperature dependence of the permeability and the appearance of a local maximum at the phase transition region could be explained in terms of a domain structure within the plane of the membranes. This explanation is based on the possibility that boundary regions between liquid and solid domains would exhibit relatively high permeability to $^{22}Na^+$.

Mixed vesicles composed of equimolar amounts of dipalmitoyl phospholipids

Abbreviations: ANS, 1-anilino-8-naphthalenesulfonic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

and cholesterol show no abrupt changes in the temperature dependence of either perylene fluorescence polarization or $^{22}Na^{+}$ diffusion rate measurements. This is taken to indicate the absence of a gross phase transition in the presence of cholesterol.

INTRODUCTION

A variety of physical techniques have recently been applied to the study of thermotropic transitions of phospholipids. It has already been established that the temperature for the solid to liquid-crystalline transition (T_c) depends largely on the chain-length and unsaturation of the acyl chains and also the chemistry of the polar head-group. Most of the relevant work has been reviewed recently[1].

The most studied phospholipid in this respect is dipalmitoylphosphatidylcholine, which undergoes a phase transition at approximately 42 °C, when in equilibrium with water in excess of 50% by weight. The endothermic reaction observed by differential scanning calorimetry at this temperature marks the onset of liquidity within the hydrocarbon region of the lamellar phase. The loss of crystallinity at the T_c has been documented by X-ray diffraction[2]. This transition is accompanied by a decrease in the thickness of the lipid membranes[2], a change in bilayer volume as observed by dilatometry[3], a marked decrease in the proton NMR line width[2,4], a decrease in the "order parameter" of ESR probes[5], a decrease in the fluorescence polarization of various probes[6–8] and an increase in the number of 1-anilino-8-naphthalenesulfonic acid (ANS) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) binding sites[5,9].

It is reasonable to expect that the solid to liquid-crystalline phase transition would have a marked effect on the permeability properties of phospholipid membranes. Moreover, such temperature-dependent changes of permeability would be of considerable physiological interest. Indeed, a number of recent studies on the temperature dependence of membrane transport and enzymatic processes have indicated the importance of phase transitions within the lipid matrix[10–15].

The present study was designed to correlate the effect of the phase transition on both the physical state and the permeability properties of phospholipid membranes. We have measured the changes in the fluorescence polarization and intensity of perylene embedded in pure synthetic dipalmitoyl phospholipid vesicles and the diffusion of sodium through vesicles of the same composition. We have also studied the effect of cholesterol and the influence of surface charge and sonication.

MATERIALS AND METHODS

Preparation of lipids

Both phospholipids used in this study were synthesized in this laboratory from purified egg yolk phosphatidylcholine[16], and contained no detectable impurities as determined by thin-layer chromatography on silica gel H and a solvent of chloroform–methanol–7 M ammonia (230:90:15, v/v/v). Dipalmitoylphosphatidylcholine was synthesized according to Robles and Van Den Berg[17], and purified on a silicic acid column. Dipalmitoylphosphatidylglycerol was synthesized from dipalmitoylphosphatidylcholine by minor modifications of the method of Dawson[18]. The fatty

acid content of purified dipalmitoylphosphatidylglycerol was 93.4% palmitic, 3.8% myristic and 2.8% stearic acid as determined by gas–liquid chromatography after methanolysis.

The procedure for the synthesis and purification of dipalmitoylphosphatidylglycerol was as follows: The substrate (1.0 mmole of dipalmitoylphosphatidylcholine) was evaporated to dryness from chloroform in a round bottom flask and suspended in 50 ml of 0.1 M sodium acetate buffer at pH 5.6. The suspension was accomplished by shaking vigorously at 50 °C in a N_2 atmosphere and the following reagents were added to the mixture: 8 ml glycerol, 30 ml of enzyme (acetone precipitated, aqueous extract from cabbage leaves[19]), 2.5 ml of 1.0 M $CaCl_2$, and 12 ml of diethyl ether. The reaction mixture was shaken under N_2 for 2 h at 30 °C. After this period, the mixture was shaken with 5 vol. of chloroform–methanol (2:1, v/v) and allowed to settle. The lower phase and interface were passed through a fluted filter paper under N_2 and the precipitate washed repeatedly with chloroform–methanol (2:1, v/v). The clear filtrate was evaporated to dryness and dissolved in 50 ml of chloroform. This solution, which contains phosphatidylglycerol, phosphatidic acid and some phosphatidylcholine, was washed once more in a chloroform–methanol–water (1.0 M NaCl and 0.1 M Na_2EDTA) system (10:5:3, v/v/v) to remove the Ca^{2+}. The lower phase was evaporated to dryness and washed again in a similar system as above, but without EDTA in the water. The final lower phase was evaporated to dryness again and dissolved in 25 ml chloroform.

Final purification of the reaction products was obtained with a 100-g silicic acid column, packed in 3% methanol in chloroform. Before the application of the lipid, 170 ml of a solution of 3% methanol in chloroform saturated with conc. NH_4OH was passed through the column (1.5 ml of conc. NH_4OH was mixed with 5 ml methanol and shaken with 165 ml chloroform. The clean lower phase was applied to the column). The inclusion of NH_4OH facilitates the separation of phosphatidylglycerol from phosphatidic acid. The lipid was applied to the column as a solution in 3% methanol in chloroform. Dipalmitoylphosphatidylglycerol was eluted with a gradient of 3% to 20% methanol in chloroform (starting volumes for a 100-g column: 780 ml 3% and 890 ml 20%). Phosphatidic acid was eluted with 50% methanol in chloroform, followed by phosphatidylcholine. All solvents were equilibrated with N_2 by bubbling immediately before use. The elution was followed by spotting a 10-μl aliquot from each 10-ml tube on a thin-layer plate and spraying with molybdenum reagent for phosphate[20]. Final yield of purified dipalmitoylphosphatidylglycerol varied from 0.3 to 0.6 mmole, or 30–60%.

Cholesterol was obtained from Sigma and recrystallized twice from methanol. All lipids were stored in chloroform under N_2 at −50 °C at a concentration of approximately 10 μmole/ml. Water was twice distilled, the second time in an all-glass apparatus. Salts and chemicals were all analytical reagent grade. *N*-Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid and histidine were obtained from Sigma. $^{22}Na^+$ was obtained from New England Nuclear. Silicic acid was obtained from Mallinckrodt (A.R., 100 mesh).

Permeability measurements

Phospholipid vesicles were made as described before[21] by a modification of the original procedure of Bangham *et al.*[22]. The dry lipids were suspended in water

containing NaCl (100 mM), histidine (2 mM), *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (2 mM), and EDTA (0.1 mM) at pH 7.4. For permeability experiments, 10 μCi of $^{22}Na^+$ (as NaCl) were included. The initial concentration of phospholipid was 5–7 μmoles/ml, estimated as inorganic phosphate after perchloric acid hydrolysis. Cholesterol was mixed with phospholipid in chloroform solution, before evaporation and addition of water. The suspension of the lipids in water was performed at approximately 42 °C, by mechanical shaking (Vortex mixer) for 10 min. Some frothing was evident at this stage, and the suspension was rather milky but stable (did not settle within 0.5 h). Mixing at room temperature did not produce stable suspensions. For experiments without sonication, the suspensions were left for 30 min equilibration at 42 °C and then cooled at room temperature for an additional h. Alternatively, the suspensions were sonicated for 1 h at 42 °C in a bath-type sonicator (Heat systems, Model 5X5, 40 kHz) with temperature control. They were then equilibrated at 42 °C for an additional 30 min and finally equilibrated at room temperature for 1 h. All the above steps were performed in a closed tube under N_2 atmosphere[21].

The equilibrated, cooled suspensions were then passed through a Sephadex column at room temperature, as before[21]. The peak containing the lipid was dispensed in 0.5-ml aliquots, and placed in dialysis bags. The bags were dialysed for 1 h at room temperature, in bulk, against 500 ml of the buffer. Finally, the bags were placed into tubes containing 10 ml of buffer and equilibrated at different temperatures in the range of 0–52 °C. The bags were transferred into new tubes at 1-h intervals. The amount of $^{22}Na^+$ present in the 10 ml dialysate at each h interval was counted in a scintillation counter and expressed as a percentage of the total $^{22}Na^+$ present at the beginning of each dialysis interval. This was taken as a measure of the self-diffusion rate of Na^+. No conversion into permeability coefficients was made, due to the lack of direct evidence that all the material was converted into single-lamellar vesicles. Gel filtration of dipalmitoylphosphatidylglycerol vesicles through Sepharose 4B indicated considerable size heterogeneity, although the captured volume (1.5 μl per μmole of phospholipid, as determined by sucrose capture) is compatible with that obtained from single lamellar vesicles of phosphatidylserine[23].

Fluorescence polarization measurements

Fluorescence polarization measurements were made in an Aminco-Bowman Spectrophotofluorimeter modified for polarization measurements according to Chen and Bowman[24]. Polarization precision was increased to ± 0.001 by averaging the photometer output using an integrating digital voltmeter. The fluorescence probe, perylene, was embedded in the membranes and excited with 410 nm radiation isolated by the excitation monochromator (12 nm band pass) and an interference filter. The fluorescence was viewed through the emission monochromator set at 468 nm (24 nm band pass) in conjunction with a cut-off filter (Corning CS3-72). Although detection of the highly polarized scattered exciting light from the membrane suspension tends to increase the measured polarization, the selected filters reduced this effect to within the precision of the measurement. Additional detail on the measurements will appear elsewhere (Jacobson, K. and Wobschall, D., unpublished).

To label the liposomes, perylene was added to the solution of dipalmitoylphosphatidylglycerol in chloroform at a molar ratio of about 1:1000 (probe/phos-

phatidylglycerol). Sonicated vesicles were subsequently produced as described above for the permeability experiments. Sonication times of 1 h were used. Final phospholipid concentrations were approximately 1.0 μmole/ml. These suspensions had an absorbance of about 0.03 at 500 nm, measured using a 1-cm path length. When cholesterol was added in a 1:1 molar ratio, an absorbance of about 0.05 was measured.

Polarizations were calculated according to the equation:

$$p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \tag{1}$$

where I_{VV} is the vertically polarized component of fluorescence and I_{VH} is the horizontally polarized component of fluorescence; the emission is excited by vertically polarized light. G is the grating transmission factor measured by the method of Azumi and McGlynn[25].

Such steady state polarizations can be converted into probe rotation rates using a recent theory by Weber[26]. In this formulation, when the rate constant describing rotation, ($\bar{R}$), is less than the rate constant for decay, (λ), and the probe is excited at a wavelength where the limiting polarization, p_0, approaches 0.5, the following equation obtains:

$$\frac{(1/p - 1/3)}{(1/p_0 - 1/3)} = (1 + 6\bar{R}/\lambda), \qquad \text{(valid for } \bar{R} < \lambda) \tag{2}$$

where $\bar{R} = (R_p + R_{0p})/2$.

R_p is the in plane rotation rate and R_{0p} is the out of plane rotation rate for a planar aromatic fluorophore. It should be noted that in a single exponential decay, the fluorescence lifetime, τ, is a measure of the several rate processes which may consume the excited state energy of the fluorophore:

$$\frac{1}{\tau} = \lambda = \lambda_e + \lambda_I + \Sigma_n \lambda_{Q_n}[Q_n] \tag{3}$$

where λ_e = rate constant for emission, λ_I = rate constant for all other intramolecular processes causing loss of excited state energy. λ_{Q_n} = rate constant for the nth bimolecular quenching processe, and $[Q_n]$ = concentration of the nth quenching species.

We calculated $\bar{R}$ as a function of temperature by measuring the polarization at various temperatures. To evaluate $\bar{R}$ from the polarization using Eqn 2, we used the limiting polarization value previously reported[27] for perylene. Excited state lifetimes for perylene-labeled dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine–cholesterol at 25 °C have also been reported[8]. It was assumed that the dipalmitoylphosphatidylcholine lifetimes would also hold for the perylene-labeled dipalmitoylphosphatidylglycerol suspensions. The lifetime was assumed to be proportional to emission intensity at 468 nm in order to obtain its temperature dependence (for this assumption to be valid, changes in spectrum shape and band position must be minimal. In fact, the spectrum of perylene is not strongly solvent sensitive and only a slight red shift ($\simeq 1$ nm) is observed when the suspension is cooled below the T_c).

RESULTS

(*A*) *Fluorescence studies*

Emission intensity. The calculated excited state lifetime of perylene in dipalmitoylphosphatidylglycerol vesicles is given in Fig. 1 as a function of temperature (the use of a single average perylene lifetime for $T > T_c$ is an approximation since the presence of more than one decay has been detected in perylene-labeled egg phosphatidylcholine liposomes above their transition temperature[8]). The lifetime changes markedly in the temperature region of the phase transition (34–40 °C) indicating that this emission intensity measurement is a sensitive indicator of the state of the phospholipid membranes. A similar abrupt increase in fluorescence intensity was observed in the region of T_c (37 °C) for perylene-labeled dipalmitoylphosphatidylcholine vesicles. However, no such abrupt change in intensity is seen with the perylene-labelled dipalmitoylphosphatidylglycerol–cholesterol liposomes; in this system the lifetime slowly increases with temperature at a rate of approximately 0.02 ns/°C.

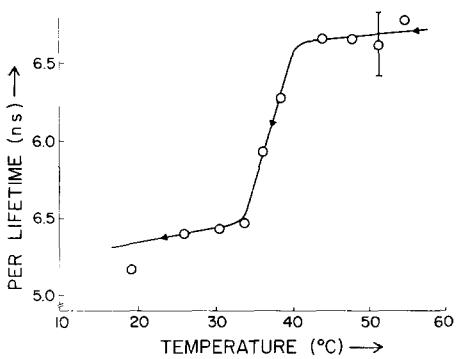

Fig. 1. Calculated perylene lifetime *vs* temperature curve for perylene-labeled dipalmitoylphosphatidylglycerol vesicles obtained on cooling suspension from 54 °C. Curve based on lifetime of perylene in dipalmitoylphosphatidylcholine at 25 °C. Estimated maximum uncertainty shown by error bar. Curve obtained on heating from $\simeq 20$ °C also displays inflection in the range 34–40 °C but does not reach as long a lifetime for $T > T_c$.

Fluorescence depolarization. Fig. 2 shows the polarization (p) *vs* temperature (T) data obtained with vesicles of pure dipalmitoylphosphatidylglycerol and a mixture of dipalmitoylphosphatidylglycerol–cholesterol (1 : 1 molar ratio), both labeled with perylene. The phase transition in dipalmitoylphosphatidylglycerol is seen as a sharp increase in the polarization as the temperature is decreased from 42 °C to 32 °C. The p *vs* T curve is almost completely reversible. However, in the region of the transition, hysteresis-like behavior is exhibited by the fact that similar p readings are displaced to 1 °C higher temperature during the melting process compared to the cooling process. In Fig. 3, the p *vs* T plots for perylene-labeled dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol vesicle suspensions are seen on an expanded scale. The polarization behavior in both systems indicates a transition in the range of 32 to 42 °C. In contrast, the p *vs* T data for dipalmitoylphosphatidylglycerol–cholesterol system (Fig. 2) gives a monotonically decreasing curve showing no hint of a structural transition in the neighborhood of 37 °C.

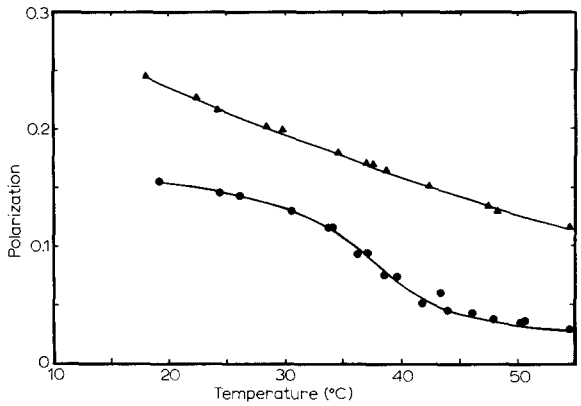

Fig. 2. Perylene polarization at different temperatures in dipalmitoylphosphatidylglycerol vesicles. (●) dipalmitoylphosphatidylglycerol at 1 μmole/ml; (▲) dipalmitoylphosphatidylglycerol (0.9 μmole/ml) mixed with cholesterol (1:1 molar ratio). Perylene/dipalmitoylphosphatidylglycerol= 1/1000. Vesicles were prepared as described in Methods, by sonication in aqueous buffered solution containing NaCl (100 mM), His-*N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (4 mM), EDTA (0.1 mM), pH 7.4.

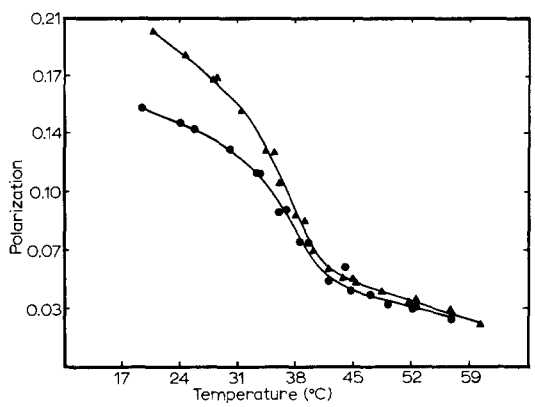

Fig. 3. Polarization *vs* temperature for perylene-labelled dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine vesicles. ●, dipalmitoylphosphatidylglycerol vesicles; ▲, dipalmitoylphosphatidylcholine vesicles (0.7 μmole/ml). Conditions for dipalmitoylphosphatidylglycerol same as in Fig. 2. Perylene/dipalmitoylphosphatidylcholine= 1/1250.

Fig. 4 shows the log $\bar{R}$ *vs* $1/T$ plots for dipalmitoylphosphatidylglycerol below and above T_c, and for dipalmitoylphosphatidylglycerol–cholesterol vesicles. From Fig. 1 and ref. 8, we assumed that the lifetime for dipalmitoylphosphatidylglycerol below T_c (20–30 °C) was constant and equal to 5.4 ns. Above the T_c (42–54 °C) we also assumed the lifetime to be constant but equal to 6.65 ns (since the variation of lifetime with temperature in ranges outside of the phase transition is much less than the approximately exponential variation of R with temperature, the calculated activation energy (E_a) is not sensitive to the selected dependence of temperature on lifetime). We see that there is a greater than 5-fold decrease in probe rotation rate in comparing dipalmitoylphosphatidylglycerol below and above the T_c. The activation energies for probe rotation on either side of the phase transition temperature are: $E_a = 9.0 \pm 1.2$ kCal/mole for $T > T_c$ and $E_a = 3.9 \pm 0.5$ kcal/mole for $T < T_c$ where the

uncertainties are the standard errors in the slopes of the Arrhenius plots. With mixed dipalmitoylphosphatidylglycerol–cholesterol vesicles, equimolar in each component, the activation energy for perylene rotation is found to be 5.6±0.1 kcal/mole.

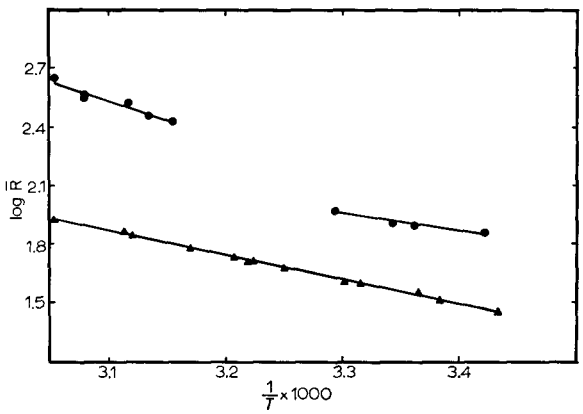

Fig. 4. Arrhenius plot of perylene average rotation rate ($\bar{R}$) in different vesicles. ●, dipalmitoylphosphatidylglycerol; ▲, dipalmitoylphosphatidylglycerol–cholesterol (1:1 molar ratio). Conditions as in Figs 2 and 3.

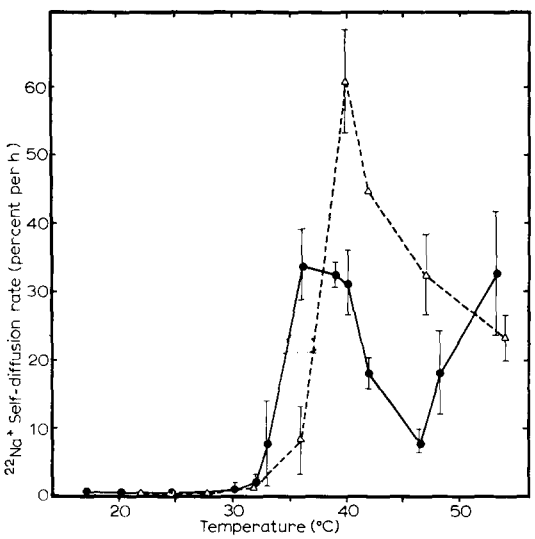

Fig. 5. Self-diffusion rates of $^{22}Na^+$ through dipalmitoylphosphatidylglycerol vesicles at different temperatures. The lipid was suspended in aqueous buffered solution at 42 °C as described in Methods. ●, vesicles produced by sonication for 1 h; △, vesicles produced by mechanical shaking. In both cases, the suspensions were cooled to 24 °C and then transferred to different temperatures. The length of vertical bars represents the spread of values obtained in different experiments, and the points the average value (8 experiments for sonicated vesicles, 2 for non-sonicated). The deviation within each experiment was usually less than 10% of the value. The standard deviation σ was calculated from the experimental points at each temperature, for the 8 experiments involving sonicated dipalmitoylphosphatidylglycerol vesicles. The σ values are given below, each preceded by the temperature (°C) and followed by the number of readings (n): (T °C, σ, n): (10, 0.01, 4); (20, 0.06, 10); (24.5, 0.2, 18); (27.8, 0.1, 6); (30, 0.6, 10); (32.5, 3.9, 12); (36, 5.2, 8); (39.5, 4.4, 6) (42, 4.7, 10); (46.5, 2.3, 10); (48, 10.5, 4); (53.2, 12.5, 4).

(B) Permeability studies

Permeability properties of dipalmitoylphosphatidylglycerol vesicles. Self-diffusion rates of $^{22}Na^+$ through dipalmitoylphosphatidylglycerol vesicles at different temperatures are shown in Fig. 5. Diffusion rates are expressed as percent of total $^{22}Na^+$ captured. The amount of $^{22}Na^+$ captured was 0.2 ± 0.05 equiv/mole of phosphate for sonicated vesicles, and approximately 0.9 equiv/mole for non-sonicated preparations. Occasionally some preparations captured much less isotope (less than one tenth of the above values) and showed relatively high self-diffusion rates and less sensitivity to temperature. This occurred in 6 out of 35 experiments and the cause for this behavior is not understood.

As shown in Fig. 5, the self-diffusion rates for $^{22}Na^+$ through sonicated dipalmitoylphosphatidylglycerol vesicles are very low below 26 °C (0.05–0.2% per h) and increase by more than two orders of magnitude in the temperature range between 26 and 36 °C. An unexpected decrease in permeability rates is then observed between 40 and 46 °C, which is reversed only at higher temperatures. Since this behavior was unexpected, the experiments were repeated several times with two different batches of dipalmitoylphosphatidylglycerol, so that the points in Fig. 5 represent averages from 8 separate experiments, 12 different temperatures (from 10 to 52 °C), and an average of 4–12 readings at each temperature. We have indicated both the total spread of values (as vertical bars in Fig. 5) and the standard deviations (as σ in the legend of Fig. 5). Most of the experiments were performed by heating the vesicles after exhaustive dialysis at 24 °C, except two, in which the vesicles were initially dialysed at 44 °C and then the experimental points were taken at lower temperatures. The maximal values in permeability at approximately 38 °C are obtained either by heating cooled vesicles or by cooling vesicles which were maintained at the original 44 °C.

Similar results have also been obtained with non-sonicated vesicles (Fig. 5). In this case the maximal permeability rate is obtained at somewhat higher temperature (40 °C), but again a significant drop is observed between 42 and 50 °C. The same behavior was observed in all experiments, in spite of considerable variation in actual rates of diffusion between experiments.

The relative diffusion rates of $^{22}Na^+$ and [^{14}C]sucrose were investigated with double-labeling experiments in which both tracers were incorporated into the same dipalmitoylphosphatidylglycerol vesicles prepared in a buffer containing NaCl (100 mM) and sucrose (10 mM). The $^{22}Na^+$ counts were estimated in a γ-counter and the ^{14}C counts in a liquid scintillation counter after subtraction of the normalized $^{22}Na^+$ counts. The results are shown in Fig. 6, where self-diffusion rates of either $^{22}Na^+$ or [^{14}C]sucrose are plotted against temperature (solid lines). The ratio of diffusion rates (Na^+/sucrose) is represented by the broken line in the same figure. It should be noted that although the rates are similar at temperatures below the T_c (ratios close to unity), there is considerable discrimination in favor of Na^+ at temperatures near the T_c (ratios approximately 2.0). When vesicles were lysed by exposing them to hypotonic solutions at 36 °C, the diffusion rates of Na^+ and sucrose were much higher (70% per h) and nearly identical in value (ratio 1.1).

Permeability properties of dipalmitoylphosphatidylcholine vesicles. As shown in Fig. 7, vesicles composed of dipalmitoylphosphatidylcholine exhibit similar behavior in terms of $^{22}Na^+$ permeability to that of dipalmitoylphosphatidylglycerol vesicles.

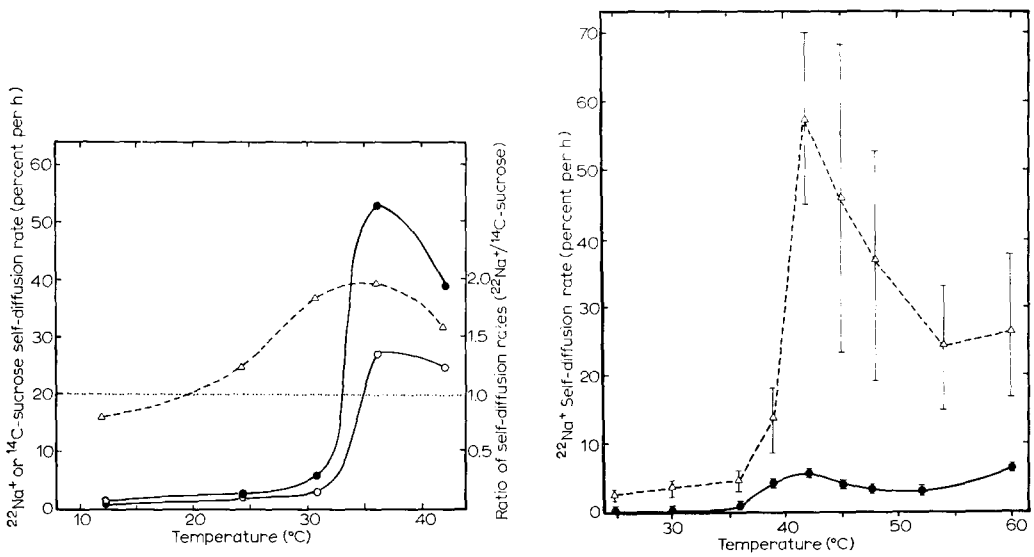

Fig. 6. Comparison of self-diffusion rates of $^{22}Na^+$ and [^{14}C]sucrose through dipalmitoylphosphatidylglycerol vesicles at different temperatures. The lipid was sonicated for 1 h at 42 °C as described in Methods, in an aqueous buffered solution containing both NaCl (100 mM) and sucrose (10 mM) and radioactive tracers $^{22}Na^+$ and [^{14}C]sucrose. The self-diffusion rates of each were measured independently in the same experiment. ●, $^{22}Na^+$; ○, [^{14}C]sucrose; △, ratio of the two self-diffusion rates ($^{22}Na/^{14}C$).

Fig. 7. Self-diffusion rates of $^{22}Na^+$ through dipalmitoylphosphatidylcholine vesicles at different temperatures. Experimental details as in Fig. 5 and Methods. ●, vesicles produced by sonication for 1 h; △, vesicles produced by mechanical shaking. The length of vertical bars represents spread of values between two individual experiments and the points the average value. Reproducibility within each experiment was better than 10% of each value.

However, some significant differences are also observed. The number of captured ions is significantly lower (0.06 ± 0.02 equiv/mole of phosphate) presumably due to the lack of net negative charge. The maximal permeability rate is obtained at approximately 42 °C, which is approximately 4 °C higher than in the case of dipalmitoylphosphatidylglycerol.

The effect of sonication, as in the case of dipalmitoylphosphatidylglycerol, is shown in Fig. 7 as producing a broadening of the high permeability peak and inducing a high permeability rate at slightly lower temperatures.

Effect of cholesterol. The addition of equimolar amounts of cholesterol has a profound effect on the permeability properties of dipalmitoylphosphatidylglycerol vesicles. As shown in Fig. 8, the presence of cholesterol produces a decrease in the diffusion rates for $^{22}Na^+$ at all temperatures. Moreover, the increase in permeability with increasing temperature shows no discontinuities and gives a linear Arrhenius plot in the range between 20 and 50 °C. The slope of the line gives an activation energy of 13 ± 0.5 kcal/mole. The Arrhenius plot of the $^{22}Na^+$ diffusion rates through pure dipalmitoylphosphatidylglycerol vesicles is also given in Fig. 8. It shows a marked deviation from linearity in the temperature range of 25 to 46 °C. These results demonstrate that cholesterol inhibits the phase-transition-induced changes in $^{22}Na^+$

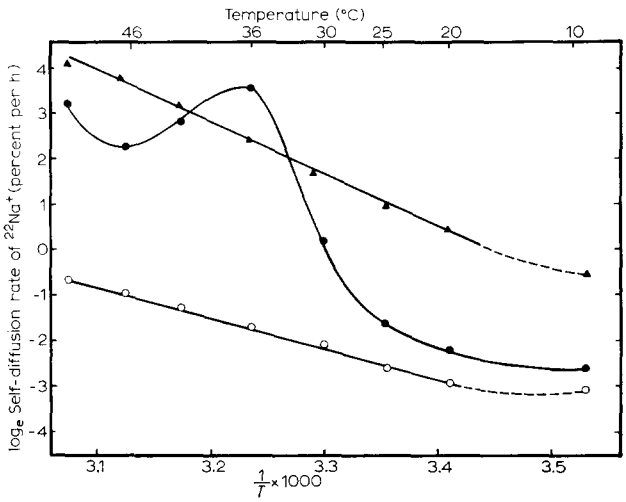

Fig. 8. Arrhenius plot of self-diffusion rates of $^{22}Na^+$ through different vesicles. Experimental conditions as in Fig. 5. All three curves were obtained with vesicles sonicated for 1 h. ●, dipalmitoylphosphatidylglycerol (averages of 5 experiments); ○, equimolar mixture of dipalmitoylphosphatidylglycerol and cholesterol (averages of 2 experiments); ▲, dioleylphosphatidylglycerol vesicles (averages of 2 experiments).

permeability of dipalmitoylphosphatidylglycerol membranes, and are in accord with the data obtained by differential scanning calorimetry[28] and the data on fluorescence polarization discussed in this report. Fig. 8 also includes the results obtained with dioleylphosphatidylglycerol vesicles. As expected from the absence of phase transitions within this temperature range[32], the Arrhenius plot for permeability is linear between 20 and 50 °C.

DISCUSSION

Fluorescence studies

At first sight the increase in emission intensity as a function of temperature (Fig. 1) may seem surprising, since emission from molecular solutions of fluorophores generally decreases with increasing temperature because rate constants for various quenching processes usually increase with temperature. To explain this behavior we propose a model in which two classes of sites are assumed to exist: one, hydrocarbon interior (Class I); and one, interfacial (Class II), as depicted in Fig. 9. We further suppose that Class I sites have a longer lifetime than Class II sites, speculating that the nature of the polar interface reduces the lifetime of probes in Class II sites relative Class I sites. In more fluid membranes, such as dipalmitoylphosphatidylglycerol above the T_c, the membrane can accomodate more impurity molecules, and because of the non-polar nature of the probe, Class I sites are favored, resulting in longer average lifetime. As the suspension is cooled below the T_c, the capacity of the hydrocarbon interior to accommodate the probe will be markedly reduced, resulting in the migration of probes to Class II sites.

The two-site model described above, is supported by the following obser-

vations: (1) measurements of perylene lifetime in various liposomal membranes by cross correlation phase fluorimetry[8] generally indicate the absence of a unique lifetime component. In other words, the probe probably resides in two or more membrane locations; however, for $T<T_c$ in dipalmitoylphosphatidylcholine membranes the measurement indicates a homogeneous population of emitters[8]. (2) Dipalmitoylphosphatidylcholine membranes below the T_c have a markedly reduced capacity to accomodate the fluorescent probe ANS (ref. 9), and the ESR probe, TEMPO (ref. 5).

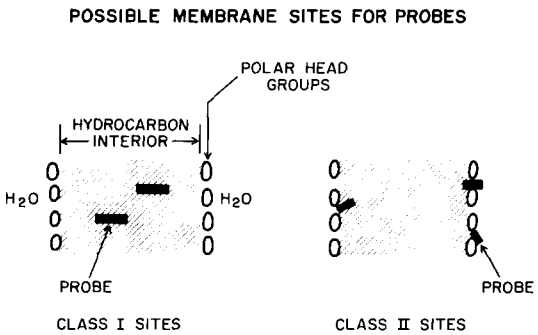

Fig. 9. Two possible classes of sites for membrane bound fluorescent probes. Class I sites are located in hydrocarbon interior. Class II sites are located in the interfacial region of the membrane.

The fluorescence polarization results are in qualitative accord with our expectations. In the perylene-labeled dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine systems, the polarization is low at temperatures where the membranes are fluid, increases sharply in the region of the liquid crystalline phase transition, and reaches high values when the membranes are solid-like. These results are in substantial agreement with recent studies[6–8]. The phase transition midpoint occurs in about the same temperature region for both suspensions, unlike the case with the permeability results (Figs 5 and 7); however, the polarization below T_c for the dipalmitoylphosphatidylcholine membranes is higher than for the dipalmitoylphosphatidylglycerol membranes. Assuming the excited state lifetime is not appreciably longer in the dipalmitoylphosphatidylglycerol membranes, the higher polarizations may reflect the fact that the neutral (dipalmitoylphosphatidylcholine) membranes display tighter molecular packing than the negatively charged (dipalmitoylphosphatidylglycerol) membranes, in which close packing is limited by electrostatic repulsion between adjacent molecules. This effect would be expected to be most pronounced in the fully condensed state, existing below the T_c.

The sharp decrease in the perylene average rotation rate below the T_c (Fig. 4) would be expected in a solid-like membrane. It seems noteworthy to mention the striking change in the rotational activation energy, E_a, from 3.9 kcal/mole for $T<T_c$ to 9.8 kcal/mole for $T>T_c$. This change may be rationalized in terms of the two site model discussed above in that the interfacial sites for probes below the T_c may provide a lower activation energy for rotation. It should also be pointed out that in solid-like membranes the energy barrier to probe rotation may be independent of temperature whereas in fluid membranes above the T_c a slight decrease in E_a with temperature will give rise to substantially higher apparent activation energies[23].

Permeability properties

The permeability properties of sonicated phospholipid vesicles, as expressed by the diffusion rates of $^{22}Na^+$ at different temperatures, show a reasonable correlation to the fluorescence studies discussed above. Thus, both measurements indicate a phase transition over similar temperature ranges as seen by a comparison of Figs 2 and 6 for dipalmitoylphosphatidylglycerol vesicles. The mid-point in the steep decrease of fluorescence polarization with temperature coincides with the peak in diffusion rates (approx. 37.5 °C), for dipalmitoylphosphatidylglycerol vesicles. Very recent experiments in this laboratory have confirmed by differential scanning calorimetry that the melting of dipalmitoylphosphatidylglycerol vesicles occurs at 38 °C (initial rise of main endothermic peak).

We tentatively conclude from this coincidence that the diffusion rate of Na^+ reaches a maximal value at the mid-point of the transition. This conclusion is strengthened by the permeability properties of dipalmitoylphosphatidylcholine shown in Fig. 7. In this case, the diffusion rate of Na^+ reaches a maximal value at approximately 42 °C, which is in agreement with the large endothermic peak obtained by differential scanning calorimetry with the same phospholipid[28].

The results given in Figs 5 and 6 contain some significant additional information. The effect of sonication is seen clearly in Fig. 5 as inducing the formation of a liquid state at a slightly lower temperature. This result could be interpreted as indicating strain in the molecular packing within sonicated vesicles.

Related to the question of molecular packing within the phospholipid vesicles, is the effect of the head-groups. A comparison of the permeability curves of dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine also shows that the dipalmitoylphosphatidylglycerol vesicles melt at a temperature approximately 4 °C lower. In this case, the presence of net negative surface charge in dipalmitoylphosphatidylglycerol and the possibility of lateral charge–charge repulsion between adjacent molecules could be inducing the observed lower melting. In contrast, it has been reported earlier that phosphatidylethanolamines melt at considerably higher temperatures compared to phosphatidylcholines with identical fatty acids[29]. In this case, intermolecular attractive interactions between amino and phosphate groups[30] (possible only in the case of phosphatidylethanolamines) would produce lateral cohesion and result in a higher T_c.

Domain effects and permeability through boundary regions

The observed maximum of self-diffusion rate of Na^+ at the mid-point temperature of the phase-transition with both dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine vesicles (Figs 5–7) is a rather surprising result. A similar local maximum has been observed in two recent studies with dipalmitoylphosphatidylcholine, involving proton relaxation times[32] and ANS fluorescence intensity[9], although it is not clear at present whether these observations are related with the maximum in permeability reported here. In an attempt to explain this phenomenon we have encountered a number of possibilities which are discussed below. Of these we favor the third, which suggests that enhanced permeability exists in local microscopic regions of disorder which are formed during the phase transitions; these regions may be envisaged as boundaries between domains.

(1) The vesicles might be undergoing an actual lysis, the rate of which is maxi-

mal at the transition mid-point due to strain in molecular packing. We have investigated this possibility by the double-labeling experiment described in Fig. 6. The results show that the self-diffusion rate of Na^+ at 30–36 °C is considerably larger than that of sucrose at the same temperature. On the other hand, when the dipalmitoylphosphatidylglycerol vesicles are deliberately lysed (osmotically) at 36 °C, the self-diffusion rates of both solutes are very similar. These two experiments strongly suggest that lysis is not the predominant cause of the permeability peak at the T_c. The possibility of a small amount of lysis, especially at low temperatures (below T_c) cannot be excluded.

(2) The maximum in diffusion rates at the mid-point of the phase transition could be due to an increased rate of vesicle fusion, but it seems rather unlikely for the following reasons. The dipalmitoylphosphatidylglycerol vesicles carry a large negative surface charge due to the ionized phosphate group which would make close contact between vesicles energetically improbable. In order to substantiate this point, we repeated the permeability experiments with dipalmitoylphosphatidylglycerol at lower ionic strength (using 10 mM NaCl buffer, instead of the usual 100 mM), and also at lower concentrations of vesicles (0.06 μmole of phosphate per ml, instead of the usual 1.5 μmoles/ml). The process of fusion should be dependent on both ionic strength (through the dependence of repulsive forces on ionic strength), and vesicle concentration (through the dependence on frequency of collisions). The results obtained under either of the above conditions were substantially identical to those reported in Fig. 5, indicating that vesicle fusion is not the predominant factor in this system.

(3) The observed maximum in diffusion rates could be related to increased permeability through microscopic regions of disorder, which are formed in the membrane plane during the process of phase transition. These regions of disorder can be obtained either transiently as single vesicles undergo a reversible solid-to-liquid transition, or as boundaries between discrete solid and liquid domains coexisting within the plane of the membrane. In any case, upon taking a time-average of the membrane structure in the vicinity of the phase transition temperature, one would find that the membrane area is divided between solid, liquid and disorder regions or domains. A mathematical analysis of the permeability of membranes with such a domain structure is given in Appendix. Under the conditions discussed below, this model predicts the observed behavior of the diffusion rates in the phase transition region.

In cases where the permeability through the boundary regions between domains is significantly greater than that through either liquid or solid domains, the curve of permeability *vs* temperature will go through a maximum around the mid-point of the phase transition, where the fractional area of boundary regions within the membrane is greatest. At temperatures slightly higher than the mid-point of the phase transition the permeability will decrease with temperature following the decrease in the fraction of boundary regions. Finally, as the membrane melts, when the suspension is heated to a temperature where the membranes are predominantly liquid-like, the diffusion rates will again increase with temperature as expected according to the Arrhenius law.

In view of the possibility that the domains in membranes may play an important part in their permeability properties, it is instructive to look for other cases where their existence may be exhibited. Evidence for co-existence of liquid and solid

domains has been obtained recently with vesicles containing a mixture of dioleyl- and distearoylphosphatidylcholine[32]. Self-diffusion rates of $^{22}Na^+$ through such mixed vesicles composed of dioleylphosphatidylglycerol and dipalmitoylphosphatidylglycerol (Papahadjopoulos, D., unpublished observation), at temperatures below the T_c for the dipalmitoylphosphatidylglycerol (0–10 °C), are considerably higher than with vesicles composed of either pure dipalmitoylphosphatidylglycerol or pure dioleylphosphatidylglycerol (Fig. 8). This observation gives support to the possibility elaborated above, that the permeability properties of a membrane composed of solid and liquid domains could be greatly affected by the boundary regions between domains. This phenomenon could be of physiological importance in relation to the function of biological membranes composed of a heterogeneous mixture of lipid components.

The effect of cholesterol

The effect of cholesterol on the properties of dipalmitoylphosphatidylglycerol vesicles is also reflected in both permeability and fluorescence polarization data. As shown in Figs 3, 4 and 8, the addition of equimolar amounts of cholesterol completely abolishes the discontinuity in both fluorescence polarization and Na^+ diffusion rates. The resulting Arrhenius plots give straight lines, in contrast to the curves obtained in the pure dipalmitoylphosphatidylglycerol. The activation energy for Na^+ diffusion is similar to that obtained with other lipids in the presence of cholesterol[23]. These results are in good agreement with the effects observed with differential scanning calorimetry (ref. 28) and other physical studies on dipalmitoylphosphatidylcholine–cholesterol dispersions, and indicate that cholesterol abolishes the phase transition of phospholipids, producing a rather rigid membrane over a wide temperature range.

The polarization obtained with dipalmitoylphosphatidylglycerol–cholesterol vesicles is considerably higher than the values obtained with pure dipalmitoylphosphatidylglycerol at these temperatures (Fig. 2). This result could be due to localization of perylene in the region of inhibited motion within the phospholipid–cholesterol bilayer[5,33]. Such localization may involve specific molecular association of perylene with cholesterol.

Physiological implications

A number of recent studies with biological membranes or whole cells have demonstrated the importance of the lipid phase-transition on some enzymatic and transport properties[10–14]. It is apparent from these studies that enzymatic and transport functions depend on the presence of a fluid lipid state, which in turn is controlled by the fatty acid chain length and *cis*-unsaturation at any particular temperature. The same phenomenon was observed recently in this laboratory with a partially purified, de-lipidized preparation of $(Na^+ + K^+)$-ATPase. In this case[15], it was shown that the enzyme could be activated by dipalmitoylphosphatidylglycerol only above the T_c. An Arrhenius plot of the enzyme activity at different temperatures exhibited a break at approximately 32 °C, which coincides with the onset of liquidity for this lipid as shown earlier in this paper. Consistent with this fluidity requirement, addition of equimolar amounts of cholesterol to dipalmitoylphosphatidylglycerol was shown[15] to inhibit completely the ability of dipalmitoylphosphatidylglycerol to activate this preparation of $(Na^+ + K^+)$-ATPase. The term viscotropic was proposed in order to describe the effect of membrane fluidity on enzyme activity[15].

It is apparent then, that phase-transition and membrane fluidity are important parameters for biological membrane function and are defined to a great extent by the chemistry of the phospholipids and the amount of cholesterol present. Changes in membrane fluidity could be involved in a number of pathological situations, including the development of early atherosclerotic lesions[34]. It is also plausible that the fluidity of membrane domains close to their phase transition points could be markedly affected by changes in parameters other than temperature, such as transmembrane electric fields. Control of certain membrane enzymatic and permeability functions could then be affected through variation of these parameters.

APPENDIX

Analysis of the temperature dependence of permeability around the phase transition temperature

In the absence of structural changes in the membrane, the expression for the permeability P_i of a substance diffusing across a membrane can be approximately written as

$$P_i = A_i \exp(-E_{a_i}/RT) \tag{1}$$

in which R is the gas constant, T is the absolute temperature, and E_{a_i} is the energy of activation. A_i is considered to be essentially temperature independent. The index i pertains to the state of the membrane and will be specified later.

The slope of the P_i *versus* T curve is

$$\frac{dP_i}{dT} = \frac{A_i E_{a_i}}{RT^2} \exp(-E_{a_i}/RT)$$

$$= \frac{E_{a_i}}{RT^2} P_i. \tag{2}$$

Let us consider in some detail a temperature interval $[T_1, T_2]$ within which the membrane undergoes phase transition from solid-crystalline to liquid-like state. We will not specify the width of this interval which may vary between a fraction of a degree to several degrees. At the temperature T_1 the plane of the membrane is a solid phase, whereas at T_2 the membrane plane is a liquid phase. At $T_1 < T < T_2$ the membrane plane is composed of patches or domains of solid and liquid. At the phase transition temperature a molecule diffusing across the membrane may encounter three possible regions: (1) solid, (2) liquid, (3) boundary between liquid and solid domains.

Let $n_1(T)$, $n_2(T)$ and $n_3(T)$ be the fractions of solid, liquid, and boundary sites, respectively.

$$n_1(T) + n_2(T) + n_3(T) = 1 \tag{3}$$

and

$$\dot{n}_1 + \dot{n}_2 + \dot{n}_3 = 0 \tag{4}$$

where the dot indicates a derivative with respect to temperature. In the following analysis we will distinguish between four temperature regions.

(a) $T<T_1$. The membrane is a solid phase. $n_1=1$; $n_2=n_3=0$; $\dot{n}_1=\dot{n}_2=\dot{n}_3=0$.

(b) $T_1<T<\overline{T}<T_2$. $\overline{T}$ is defined as a temperature below which the fraction of boundary regions n_3 is increasing. $\dot{n}_1<0$; $\dot{n}_2>0$; $\dot{n}_3>0$.

(c) $\overline{T}<T<T_2$. In this region, the fraction of boundary regions is decreasing. $\dot{n}_1<0$, $\dot{n}_2>0$, $\dot{n}_3<0$.

(d) $T_2<T$. The membrane plane is a liquid phase. $n_1=n_3=0$, $n_2=1$; $\dot{n}_1=\dot{n}_2=\dot{n}_3=0$.

The expression for the permeability is

$$P(T)=\sum_{i=1}^{3} A_i n_i \exp(-E_i/RT) \tag{5}$$

The slope is (see also Eqn 2)

$$\dot{P}=\sum_{i=1}^{3} (P_i n_i E_{ai}/RT^2+P_i\dot{n}_i) \tag{6}$$

According to Eqns 3 and 4, n_1 and $\dot{n}_1$ may be expressed in terms of n_2, n_3 and $\dot{n}_2$, $\dot{n}_3$. Hence

$$P=\frac{1}{RT^2}[E_{a1}P_1+n_2(E_{a2}P_2-E_{a1}P_1)+n_3(E_{a3}P_3-E_{a1}P_1)]+$$
$$\{(P_2-P_1)\dot{n}_2+(P_3-P_1)\dot{n}_3\} \tag{7}$$

From inspection of the data on diffusion in solids and liquids[35] and the data on diffusion and permeability in liquids and polymers[36,37] it follows that $P_2>P_1$ and $P_3>P_1$. The explanation for this is that diffusion coefficients and permeability coefficients increase with the increase in mobility of the matrix[38–42] when moving in the sequence: solid, crystalline and glassy polymers, liquids. In the following treatment we will further assume that $P_2\,E_2>P_1\,E_1$ and $P_3\,E_3>P_1\,E_1$. This mathematical assumption can be justified because of the exponential dependence of P on E, and in view of the observed inequality, $E_a>RT$.

In Region b the slope is larger than in Region a due to the addition of several positive terms to the first term $E_{a1}P_1/RT^2$ which is the slope in Region a. The increase in the slope may be quite significant due to the terms in the curly brackets. If the phase transition occurs over a narrow temperature interval, there will be abrupt changes in the n_i values and the slope $\dot{P}$ may show a very steep increase in the Region b. Note that when the phase transition is abrupt the second term in the curly brackets, which depends on temperature derivatives is the dominant term. Consider now Region c. The terms in the square brackets in Eqn 7 are positive. The term $(P_2-P_1)\,\dot{n}_2$ in the curly brackets is positive whereas the term $(P_3-P_1)\,\dot{n}_3$ is negative. If P_3 is sufficiently greater than P_2 it can happen that the slope will be negative, *i.e.*, the permeability will decrease with temperature in Region c. In Region d the permeability will again increase with temperature according to the Arrhenius law. Thus, for certain ranges of the permeability values (P_1, P_2, P_3) this model will predict

the cation permeability behavior observed in phospholipid vesicles undergoing a phase transition.

NOTE ADDED IN PROOF (Received May 14th, 1973)

A recent study of the effects of fast cooling on the permeability properties of *E. coli* and liposomes has also indicated that lowering the temperature below the T_c may produce discontinuities in packing, resulting in release of small molecules[43].

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