Phase Transitions and Phase Separations in Phospholipid Membranes Induced by Changes in Temperature, pH, and Concentration of Bivalent Cations†

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ABSTRACT: Differential scanning calorimetry (DSC) and fluorescence polarization of embedded probe molecules were used to detect phase behavior of various phospholipids. The techniques were directly compared for detecting the transition of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidic acid (DPPA) dispersed in aqueous salt solutions. Excellent agreement occurred in the case of phosphatidylcholine; however, in the case of phosphatidic acid, at pH 6.5, transitions detected by fluorescence polarization using the disc-like perylene molecule occurred about 10° lower than those detected by DSC. Discrepancy between fluorescence and DSC methods is eliminated by using a rod-like molecule, diphenylhexatriene (DPH). Both techniques show that doubly ionizing the phosphate group reduces the T_c by about 9°. Direct pH titration of fluidity can be accomplished and this effect is most dramatic when membranes are in their transition temperature range (*ca.* 50°). Phosphatidic acid transitions occur at higher temperatures, and have appreciably lower transition enthalpies and entropies than phosphatidylcholine. These effects could not be explained simply on the basis of double layer electrostatics and several other factors were discussed in an attempt to rationalize the results. Addition of monovalent cations (0.01–0.5 M) is shown to increase the T_c of dipalmitoylphosphatidylglycerol by less than 3°. However, addition of (1×10^{-3} M) Ca^{2+} abolishes the phase transition of both phosphatidylglycerol and phosphatidylserine in the range 0–70°. Preliminary X-ray evidence indicates the phosphatidylserine–Ca^{2+} bilayers are in a crystalline state at 24°. In contrast, 5×10^{-3} M Mg^{2+} only broadens the transition and increases the T_c indicating a considerable difference between the effects of Ca^{2+} and Mg^{2+}. Neutralization of PS increases the T_c from 6° (at pH 7.4) to 20–26° (at pH 2.5–3.0) but does not abolish the transition, suggesting the Ca^{2+} effect involves more than charge neutralization. Addition of Ca^{2+} to mixed phosphatidylserine–phosphatidylcholine dispersions, induces a phase separation of the dipalmitoyl- (and also distearoyl-) phosphatidylcholine as seen by the appearance of a new endothermic peak at 41° (58°). Similarly, in mixed (dipalmitoyl) phosphatidic acid–phosphatidylcholine (2:1) dispersions, Ca^{2+} again can separate the phosphatidylcholine component.

The gel to liquid crystalline phase transition in phospholipid bilayers may be described as a highly cooperative, order-disorder transition in which the bilayer undergoes a lateral expansion and a decrease in thickness and density (*cf.* Oldfield and Chapman, 1972; Nagle, 1973a,b). Hydrocarbon chain packing changes from all-trans below the transition temperature (T_c) to a state in which the chains probably have from 7 to 12 gauche rotational isomers above the T_c for dipalmitoylphosphatidylcholine (DPPC)[1] bilayers. The state of molecular packing in phospholipid bilayers can be affected by the lipid component(s), the temperature, and ionic composition of the aqueous environment. The sensitivity of a given bilayer structure to the composition of the aqueous solution is particularly interesting physiologically, and it depends strictly on the chemical nature of head group (Träuble and Eibl, 1974; Michaelson *et al.*, 1974; Papahadjopoulos and Ohki, 1969). A number of studies have demonstrated that the physical state of the phospholipid acyl chains can markedly affect the activity of membrane transport processes (Wilson *et al.*, 1970; Overath *et al.*, 1970; Esfahani *et al.*, 1971) and membrane enzymes (reviewed by Raison, 1973) according to what has been termed a "viscotropic" effect (Kimelberg and Papahadjopoulos, 1972, 1974).

In this study, we employed the techniques of differential scanning calorimetry (DSC) and fluorescence polarization to study phase transitions and phase separations in phospholipid vesicles. First, a correlation between the two techniques was established using a well-studied system: aqueous dispersions of DPPC. Comparisons of the melting behavior of DPPC, DPPG, DPPA, and PS were made. The effects of pH, monovalent, and divalent cations on the melting behavior of the acidic phospholipid vesicles and on the phase separation characteristics of mixed lipid vesicles were studied.

Materials and Methods

Preparation of Lipids. The phospholipids used in this study were synthesized in this laboratory 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). Dipalmitoylphosphatidylcholine (1,2-dihexadecyl-*sn*-glycero-3-phosphorylcho-

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[1] Abbreviations used are: DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DPPA, dipalmitoylphosphatidic acid; DPPS, dipalmitoylphosphatidylserine; PC, egg phosphatidylcholine; PS, beef brain phosphatidylserine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Per, perylene; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimeter; T_c, phase transition midpoint temperature; ΔT, transition width in temperature; p, fluorescence polarization; T_1, temperature of transition onset; T_2, temperature of transition end; $\bar{R}$, average probe rotation rate.

line; DPPC) was synthesized according to Robles and Van Den Berg (1969) and purified on a silicic acid column. The analysis of fatty acid esters indicated more than 99% palmitic acid, the rest being myristic and stearic. Dipalmitoylphosphatidylglycerol (DPPG) was synthesized from DPPC by minor modifications of the method of Dawson (1967), and further purified on silicic acid (Papahadjopoulos *et al.*, 1973a). Dipalmitoylphosphatidic acid (DPPA) was produced from DPPC by enzymatic cleavage of the choline group using phospholipase C and purified on silicic acid (Papahadjopoulos and Miller, 1967). The preparation was washed exhaustively with EDTA to remove Ca^{2+}. Phosphatidylserine (PS) was purified from beef brain (Papahadjopoulos and Miller, 1967). The phospholipids were stored as solutions in chloroform (10–20 μmol/ml) in sealed ampoules, under nitrogen, at −50°.

Other Chemicals. Palmitic acid used for the synthesis of the phospholipids was from Fluka AG, Switzerland (puriss, >99%). Perylene (Gold Label) and diphenylhexatriene (98%) were purchased from Aldrich. L-Histidine (Sigma grade) and TES were obtained from Sigma Chemical Co. (St. Louis, Mo.). Silicic acid was from MallincKrodt (A-R, 100 mesh). All solvents and chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus.

Lipid Dispersions. Phospholipid vesicles (liposomes) were made as previously described (Bangham *et al.*, 1965; Papahadjopoulos, 1970).

The chloroform solution containing purified lipid from a newly opened ampoule was transferred to a glass tube which had been flushed with high purity nitrogen (Linde, H. P., Dry 99.996%). The chloroform was then evaporated under vacuum. The dry lipids were suspended in buffers containing NaCl, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 2 mM L-histidine, and 0.1 mM EDTA by mechanical shaking on a Vortex mixer for 10 min under nitrogen. DPPC and DPPG were suspended and sonicated (where stated) at 42°. In the case of DPPA, dispersions at pH 9.0 were formed by shaking at about 60–65°, while dispersions at pH 6.0 were formed by shaking at about 68°. Sonication, when required, was performed at 58° for 45 min. In the case of the fluorescence experiments where pH was varied, uncoupler S-13 was added to DPPA in molar ratio 1:2000 (Nicholls and Wenner, 1972) to ensure rapid pH equilibration across the lamellae. Sonication was performed in a bath-type sonicator under nitrogen, as before (Papahadjopoulos, 1970).

Differential Scanning Calorimetry. The transition temperatures (T_c) of the phospholipid dispersions were determined with a differential scanning calorimeter (Perkin-Elmer DSC-2), calibrated with Indium. The lipid samples contained 0.4–0.7 μmol of phosphate in 15 μl; they were prepared and dispersed in the same buffer (1 μmol/ml) as described for the preparation of vesicles. (Unless specifically stated, the samples were not sonicated.) The suspensions were then centrifuged at 100,000*g* for 30 min at 25° and the wet pellets transferred to the calorimeter pans. The heating rate[2] was usually 5°/min. Transition enthalpies (ΔH) were determined after measuring the area under the excess specific heat curve by paper weighing. Phospholipid was estimated directly in the sample by phosphate assay after calorimetry. All experiments were repeated on at least two to four separate preparations. To examine sonicated vesicles by DSC and fluorescence, sonicated samples were centrifuged at 100,000*g* for 1 hr at 20° to remove big liposomes. About 10–20% of the total phosphate comes down in the pellet. Supernatants were used as such for polarization studies but they were concentrated by ultra filtration for 5 hr at room temperature for DSC measurements. When the turbidity (τ) was measured as a function of wavelength (λ) in the range of 450–250 nm, a plot of log τ *vs.* log λ was linear with a slope of about −4. This indicates a suspension of particles, scattering according to Rayleigh's law, whose diameter is *ca.* 400 Å or less.

Fluorescence Measurements. Vesicles were labeled in the manner previously described (Jacobson and Wobschall, 1974; Papahadjopoulos *et al.*, 1973a). Suspensions were equilibrated at temperatures above the T_c for at least an hour and then slowly cooled to room temperature before the upward temperature scan. Fluorescence intensity and polarization measurements were made as previously described (Jacobson and Wobshall, 1974) except that a ratio recording system to compensate for exciting beam fluctuations and a ratio averaging photometer with digital readout were employed (SLM Instruments, Champaign, Ill.).

Our approach to minimizing the error introduced by using turbid, nonsonicated lipid dispersions was simply to reduce the cuvet size to 5 and 2 mm (Precision Cells, Hicksville, N.Y.). Other approaches have been employed (Faucon and Lussan, 1973). Adapters to seat the smaller cells were machined from brass and a thermistor temperature sensor was inserted directly into a port in the brass cuvet block and surrounded with propylene glycol to ensure thermal contact. We found the smaller cells to be useful even for the less turbid sonicated suspensions as, for example, the polarization of perylene embedded in sonicated DPPC vesicles at 24° is about 10% higher in the 2-mm cuvet than the 5- or 10-mm cuvet. Since the background light scattering error was negligible, this result indicated a slight depolarization artefact in the larger cells in spite of relatively little turbidity (OD with 10-mm path at 500 nm = 0.14; [DPPC] = 1 μmol/ml). Reducing DPPC concentration to 0.2μmol/ml practically eliminated the differences in polarization between cuvets. With the 2-mm cuvet 1-mm excitation and emissions slits were employed. In the Aminco fluorimeter, 1 mm of slit width is equivalent to 12-nm bandpass (Chen, 1967).

Perylene was excited at 410 nm and emission measured at 470 nm through 3 mm of aqueous 2 M $NaNO_2$ and a Corning CS3-72 filter. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was excited at 365 nm using the excitation monochromator and an interference filter and emission observed at 460 nm through 3 mm of aqueous 2M $NaNO_2$. Rapid loss of signal occurs upon excitation presumably due to a reversible photoisomerization, but is minimized by exposures less than 10 sec (Shinitzky and Barenholz, 1974).

Several methods were used to estimate transition midpoint (T_c). A midpoint was obtained by estimating the transition width in temperature and taking its midpoint as T_c (Papahadjopoulos *et al.*, 1973a). Secondly, the graphical method of Träuble and Overath (1973) was employed. In addition, the first derivative plots of the polarization and intensity *vs.* T curves were produced by evaluating the slope of the line connecting successive data points. Maximum values of the slope in these plots were taken as the T_c, and

[2] Heating at slower rates (2.5, 1, and 0.5°/min) allows linear extrapolation to zero heating rate. With DPPC, such an extrapolation shows that the T_c is decreased by less than 1° by heating infinitely slowly.

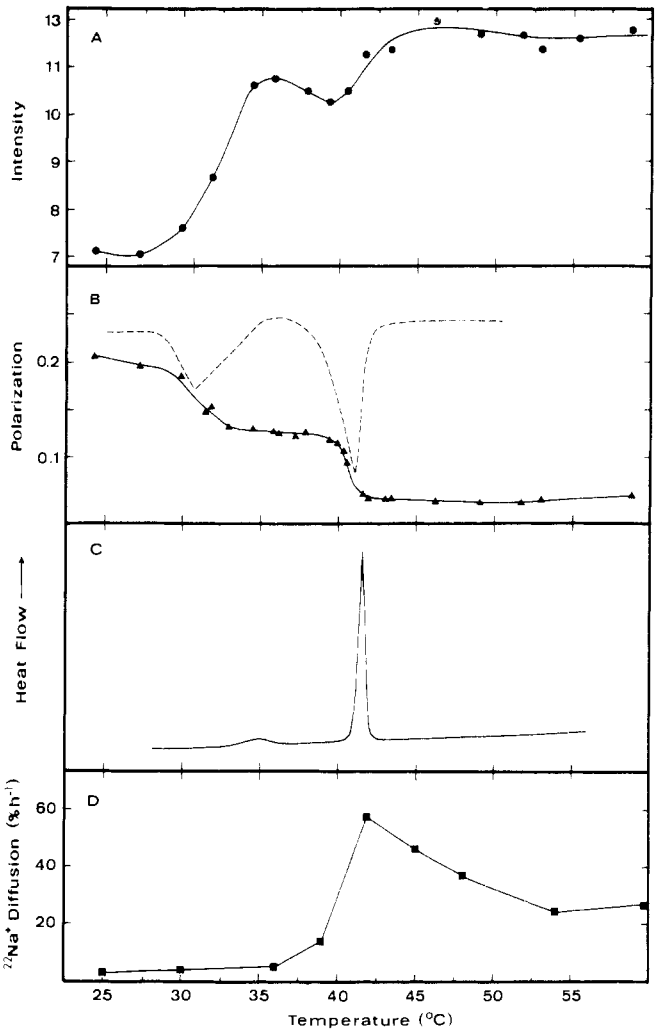

FIGURE 1: Phase transition of DPPC vesicles. (A) Intensity of perylene fluorescence (arbitrary units) at various temperatures in DPPC dispersion (Per/DPPC < 1:666; [DPPC] = 0.8 μmol/ml). (B) Polarization of perylene embedded in DPPC dispersions as in (A); broken line represents first derivative of p-T scan, evaluated as described in text. (C) Differential scanning calorimeter thermogram for DPPC dispersion; heating rate, 1.25°/min. (D) $^{22}Na^{2+}$ self-diffusion rate through unsonicated DPPC vesicle membrane at various temperatures (efflux expressed as per cent of captured ions per *h*; Papahadjopoulos *et al.*, 1973a). All dispersions prepared in 100 mM NaCl-4 mM His-TES-0.1 mM EDTA at pH 7.4, without sonication.

all slope values were assigned to the average temperature between the two original data points. The three methods agreed to within 0.4° in the estimation of T_c with unsonicated DPPC vesicles and to within 1.5° on sonicated DPPC dispersions. Temperature accuracy is estimated at ±0.2°. All computations and preliminary plotting were done by computer.

Any absorption or turbidity measurements were made with a Cary 14 spectrophotometer.

Results

Several Physical Measurements of the Phase Transition in DPPC Vesicles and Comparison of Results. Figure 1B (solid line) shows the polarization curve for perylene embedded in unsonicated DPPC dispersions. The polarization (p) drops sharply in the region of the main transition (*ca.* 41°). The width of the transition (ΔT) is less than 1.5° and, thus, sharper than previously reported transitions detected by fluorescence (Faucon and Lussan, 1973). Furthermore, this measurement also apparently reveals the pretransition or minor transition behavior as another steep drop in polarization at approximately 31°. This is interesting because the minor transition has not previously been detected by fluorescence polarization in unsonicated vesicles (Faucon and Lussan, 1973). This behavior is also clearly seen in the first derivative plot (dp/dT) of p *vs.* T (Figure 1B, dotted line). Assuming the limiting polarization and lifetime are not markedly altered in these transitions, the sharp drops in polarization correspond to marked fluidity increases in the environment of the probe. If one computes the average rotation rate of the probe ($\bar{R}$) as described previously (Papahadjopoulos *et al.*, 1973a) $\bar{R}$ increases by 90% going through the main transition as contrasted to a 25% increase in $\bar{R}$ through the minor transition.

For our transition studies we have chosen to present heating scans to avoid possible super cooling effects. Indeed, as reported earlier (Papahadjopoulos *et al.*, 1973a), cooling scans are displaced about 1° lower in temperature than heating scans. In addition, the minor transition was not as easy to detect on cooling, in agreement with Sackmann *et al.* (1973) and Yi and MacDonald (1973).

Perylene fluorescence intensity generally increases on melting of the DPPC dispersion, but it is interesting (Figure 1A) that a local maximum in fluorescence intensity occurs in the region of the minor transition although at slightly greater temperature (33–35°) than the change in polarization. While polarization heating and cooling scans showed good agreement, especially at and above the main transition, intensity scans were not reversible when the sample was cycled from high to low to high temperatures in agreement with the turbidity data of Yi and MacDonald (1973, Figure 7). This observation may indicate aggregation (Yi and MacDonald, 1973). As shown in Figure 1C and D, the transitions observed by perylene fluorescence are in good agreement with the results obtained by scanning calorimetry and also by following the efflux of Na^+ through the same vesicles. Sonication followed by centrifugation to remove big liposomes broadens the transition ($\Delta T \simeq 9.5°$) and lowers the T_c to about 37–39° as is to be expected (Sheetz and Chan, 1972).

Table I gives a comparison of DPPC melting behavior as detected by several different physical techniques. Density dilatometry (Nagle, 1973a) gives the sharpest main transition with an apparent midpoint of about 41.5, in good agreement with our DSC results. The two probe methods (rows 4 and 5) report the transition onset at slightly lower temperatures. In both of these methods, the probe is thought to be largely excluded from the hydrocarbon region below the T_c. The minor transition was explicitly detected by all methods except permeability although in this case it may be part of the increasing permeability seen in the range 36–39° (Figure 1D). In contrast to the main transition, the estimated midpoints of the minor transition vary from 29.5 to 37.5° with the probe methods giving the lower values. Whatever structural alteration occurs at the pretransition it is reported in both probe methods to be on the order of 25–40% of the main transition response while the "intrinsic" methods report it to be about 10–20% of the main transition (Hinz and Sturtevant, 1972; Nagle, 1973a,b; Table II). This disproportionate probe response suggests that probe segregation is associated with the minor transition.

DSC and Fluorescence Studies of Phase Transitions in Dipalmitoyl Phospholipids with Different Head Groups. Figure 1C shows the thermogram for DPPC and Figure 5a,

TABLE I: Results of Several Methods for Detecting Transitions of Aqueous DPPC Dispersions.[a]

Method	Main Transition Temp (°C)				Minor Transition Temp (°C)			
	Midpoint (T_c)	Onset (T_1)	End (T_2)	ΔT ($T_2 - T_1$)	Midpoint (T')	Onset (T_1')	End (T_2')	$\Delta T'$ ($T_2' - T_1'$)
DSC[b]	41.1 ± 0.2	40.7	41.5	0.8	37.5	34.5	39.0	4.5
Density dilatometry[c]	41.5	41.3	41.8	0.45	34.1	32.8	35.6	2.8
Permeability[d]	40–45	36–39				None	detected	
Fluorescence polarization[e]	41 ± 0.2	40.2	41.9	1.7	31.6	30–30.5	32.7	2.2–2.7
Spin-label partitioning[f]	40.5	40	41.5	1.5	29.5	27.5	31.0	3.5

[a] All dispersions were obtained by equilibration in excess water, without sonication. [b] T_c, T_1, and T_2 obtained by extrapolation to zero heating rate using a linear plot of values obtained at 0.6, 1.2, 5, and 10°/min; values for T_1 and T_2 obtained by extrapolation of ascending and descending arms of main peak through base line; ΔT such that 99% of enthalpy of fusion is contained. Primed values refer to temperatures characterizing the minor transition. [c] Nagle, 1973. [d] Papahadjopoulos *et al.*, 1973a. [e] Midpoint values stated are averages of three methods of estimation as given under Materials and Methods. [f] Shimshick and McConnell, 1973.

TABLE II: Phase Transition Data for Various Dipalmitoyl Phospholipid Dispersions.[a]

Phospholipid (pH)	T_c (°C)	T_1 (°C)	ΔT (°C)	ΔH (kcal/mol)	ΔS (cal/mol °K)
DPPC (7.4)[b]	42.4 ± 0.2	41.4	3.6	7.7 ± 0.5	24
DPPC (7.4)[c]	37 ± 1			1.2 ± 0.2	
$DPPG^-$ (7.4)[b]	41 ± 0.2	39.9	4.0	7.9 ± 0.4	24
$DPPG^-$ (7.4)[c]	35 ± 1			0.5 ± 0.2	
$DPPA^-$ (6.5)[d]	67 ± 1	63 (57)	7 (13)	5.2 ± 0.2	15
$DPPA^{2-}$ (9.1)[d]	58 ± 1	52 (50)	11 (16)	2.9 ± 0.5	9

[a] Calorimeter heating rate, 5°/min; T_1 is temperature at which the predominant slope of ascending arm of the major endothermic peak intercepts base line; ΔT is the temperature interval which includes 90–95% of the transition enthalpy and is calculated from the intercepts of the main slopes to the ascending and descending arms of the endothermic peak with the base line. Values in parentheses are the points at which endothermic peak deviates from base line. All values given are averages obtained from 4 to 12 separate experiments. Conditions are described under Materials and Methods, in NaCl (100 mM) buffered at the indicated pH. The difference in T_c of DPPC given here with that in Table I is due to differences in heating rates as indicated in both tables and in text. [b] Main transition. The values in kcal/mol for the total ΔH (both minor and main transitions) are 8.9 for DPPC, in reasonable agreement with Chapman *et al.* (1974), and 8.4 for DPPG. [c] Minor transition. The values for T_c and ΔH for this transition are diminished considerably following consecutive heating and cooling. Values given are from first runs. [d] From a single broad transition; no minor transition was observed with DPPA.

the thermogram for DPPG at pH 7.4. Thermograms for DPPA at pH 6.5 and 9.1 are given in Figures 2C, and 3C, respectively, and indicate a difference of approximately 10° in T_c. Table II gives a summary of the transition parameters obtained from calorimeter data for several dipalmitoyl phospholipid bilayers. ΔH is the enthalpy and ΔS is the entropy of transition calculated on the basis that the transition is first order (Phillips *et al.*, 1969). In the case of DPPA vesicles, the increase in surface charge density induced by a change in pH from 6.5 to 9.1 is seen to markedly decrease the transition enthalpy and entropy. Our values for ΔH of the main transition of DPPC are 20% lower than those reported by Hinz and Sturtevant (1972), although a revised figure, 9.0 kcal/mol (Dr. J. M. Sturtevant, personal communication), reduces the difference to 15%.

Figure 2A and B shows measurements of the fluorescence polarization (circles) and intensity (triangles) of perylene, embedded in sonicated and nonsonicated vesicles, respectively, as a function of temperature. (The dotted line in Figure 2B shows the polarization for the probe DPH embedded in unsonicated DPPA vesicles at pH 6.5.) Exactly similar plots for DPPA at pH 9.1 are given in Figure 3A and B. In agreement with the DPPC results, melting is associated with a decrease in polarization. Fluorescence intensity is low when the membranes are in the gel state and increases markedly as the bilayers melt by amounts dependent on the pH and whether sonicated or not. The low intensity in the gel state is thought to be due to expulsion of the probe from the hydrocarbon region to a position in proximity to the head groups where quenching can occur (Papahadjopoulos *et al.*, 1973a). A second possibility is that a concentration quenching effect exists below the T_c due to the occurrence of probe (impurity) pools formed, perhaps, near the bilayer center plane. However, since the amount of quenching is markedly dependent on lipid head groups, this would not seem as likely as the first hypothesis.

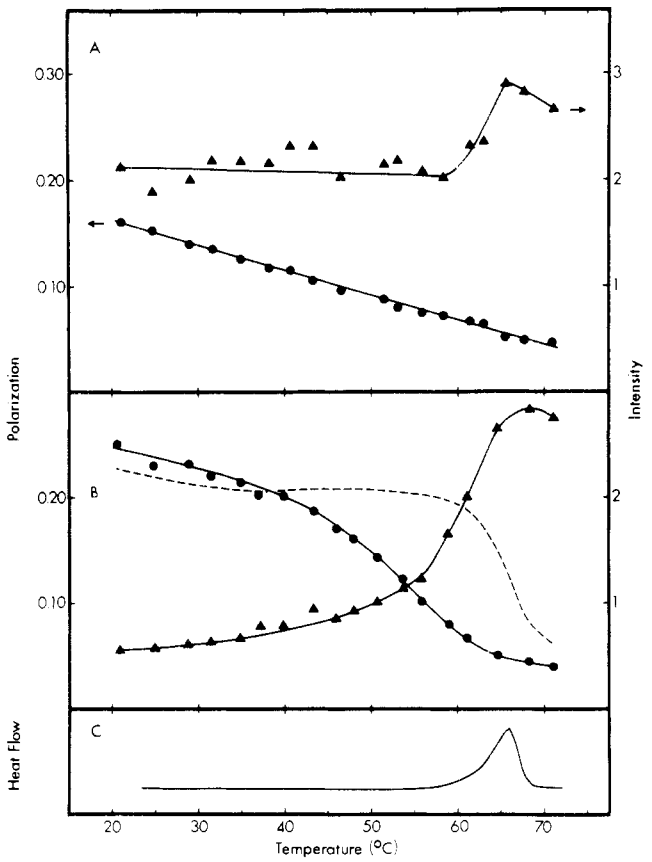

FIGURE 2: Phase transition of DPPA vesicles at pH 6.5. (A) Left ordinate (●) p of perylene embedded in sonicated DPPA dispersion; right ordinate (▲) intensity of Per fluorescence (arbitrary units) from same dispersion (Per/DPPA 1:500; [DPPA] = 0.8 μmol/ml). (B) same as (A) except dispersion is not sonicated; (- - -) p of DPH embedded in DPPA dispersions at pH 6.5 as above (DPH/DPPA = 1:875; [DPPA] = 1.25 μmol/ml; note: the scale for DPH p values is double that shown). (C) DSC thermogram for DPPA dispersion at pH 6.5; heating rate, 5°/min. All dispersions prepared in 10 mM NaCl–4 mM His-TES-1.0 mM EDTA at pH 6.5 except that buffers for Per-labeled dispersions were 2 mM in NaOAc as well and had uncoupler, S-13, added at 1:2000 (S-13/DPPA).

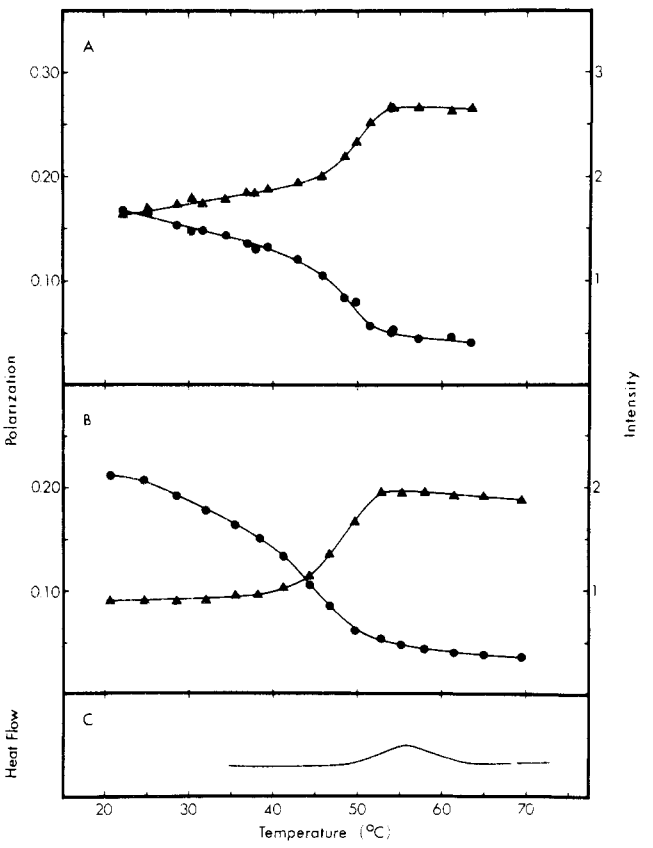

FIGURE 3: Phase transition of DPPA vesicles at pH 9.1. Notations and conditions are exactly the same as in Figure 2 except that pH is 9.1.

Only heating scans are shown; cooling typically results in a decrease of a few degrees in the apparent transition. Thermograms show broad transitions ($\Delta T \simeq 15°$) at both pH values. Both fluorescence parameters indicate that the T_c decreases by approximately 10° when pH is raised from 6.5 to 9.1, the expected region for the second pK of DPPA.

In contrast to DPPC, the fluorescence curves with perylene are displaced to appreciably lower temperatures and are broader compared to the transitions obtained with DSC (*e.g.*, for unsonicated DPPA at pH 6.5, $T_c = 67 \pm 1°$ by DSC and approximately 55° by fluorescence polarization and 58° by fluorescence intensity). On the other hand, DPH polarization reports the transition midpoint at pH 6.5 to occur at 66.7° in excellent agreement with the DSC result (67 ± 1°). The transition widths measured by the two techniques are also similar.

Sonication brings anomalous results as reported by perylene: (a) no transition is detected by polarization at pH 6.5; (b) both polarization and intensity reveal the transition temperature to be increased slightly whereas transition widths are narrowed to 6–8°. Since polarization depends on the ratio of probe rotational relaxation time to its fluorescence lifetime, compensating changes in this ratio could occur so that either polarization or intensity alone may not be accurate indicators of the phase transition. Probe rotation rate was therefore calculated (Jacobson and Wobschall, 1974; Papahadjopoulos *et al.*, 1973a) based on the assumption that the lifetime of perylene in a fluid membrane is 6.0 nsec (Cogan *et al.*, 1973; Lanyi, 1974) and is proportional to total intensity. These rotation rate–temperature plots do show the transitions for both sonicated and unsonicated vesicles, although they are broad (approximately 20°) and show little difference in the T_c between pH 6.5 and 9.1. It appears then that in sonicated DPPA dispersions at pH 6.5, lifetime and rotation rate do change in a compensating way so that polarization alone does not adequately reflect the transition.

Effect of pH Changes on DPPA Fluidity. Figure 4A and B show pH titration of perylene polarization in sonicated and unsonicated DPPA vesicles, respectively, at various temperatures. If the suspensions are held in their transition temperature range, an increase in the pH from 6 to 10 results in a sharp drop in polarization between pH 8.0 and 9.5, suggesting that the second ionization of the phosphate moiety can "melt" the membranes. The apparent pK_2 for the ionization is approximately 8.7 in rough agreement with previous results (Papahadjopoulos, 1968) with egg phosphatidic acid monolayers. During titration at $T > T_c$, changes in fluorescence polarization are diminished in unsonicated dispersions and absent in sonicated suspensions (Figure 4A and B). In sonicated vesicles, no titration behavior is observed below the transition. However, the molecular packing below the T_c in unsonicated dispersions is apparently sensitive to pH. It is evident from the above results, that the most dramatic changes in fluidity induced by pH are obtained for membranes at temperatures within their transition regions.

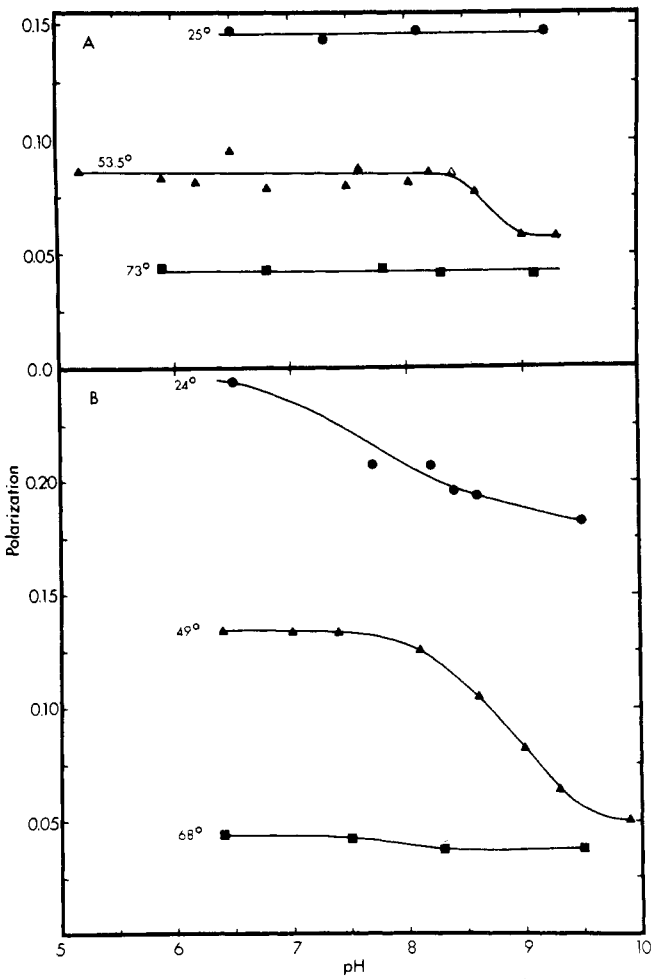

FIGURE 4: pH titration of polarization of perylene embedded in DPPA vesicles at various temperatures. All dispersions prepared in 10 mM NaCl–4 mM His-TES–2.0 mM NaOAc–0.1 mM EDTA. (A) Sonicated DPPA dispersions; (B) unsonicated DPPA dispersions. Other conditions similar to Figures 2 and 3.

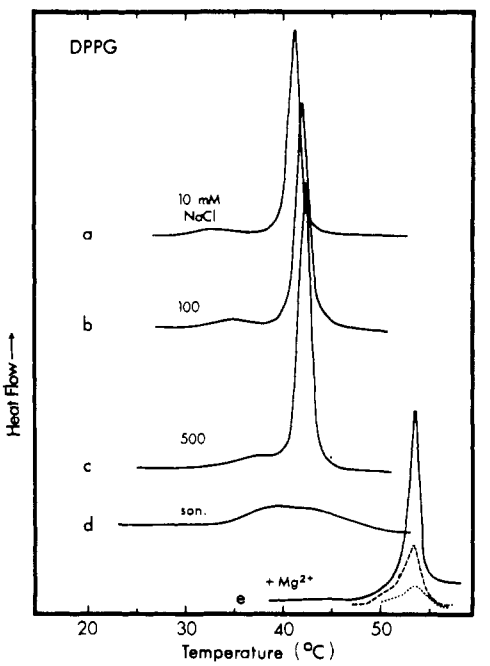

FIGURE 5: Differential scanning calorimetry for DPPG. Heating rate, 5°/min. Thermograms(a–c) for DPPG at NaCl concentrations of 10, 100, and 500 mM, respectively; (d) thermogram for sonicated DPPG in 100 mM NaCl; (e) same as in (d) after subsequent addition of 10 mM Mg^{2+}; (- - -) and (. . .) indicate effect of successive heating scans, respectively. Details of sample preparation, as under Materials and Methods.

Effect of Bivalent Metals and Ionic Strength on Phase Transitions. We have investigated the effect of changes in ionic strength on the properties of dipalmitoylphosphatidylglycerol (DPPG) as measured by the differential scanning calorimeter. The results, shown in Figure 5 (curves a–c), indicate a shift of the main endothermic peak in higher temperature (by 1°, from 41 to 42°), as NaCl concentration was increased from 10 to 100 mM, and a further increase of 0.5° at 500 mM. A concomitant shift to higher temperatures (by 4.5°) was also observed for the midpoint of the minor transition. Similar experiments with DPPG in different concentrations of KCl gave substantially the same results. When DPPC was dispersed in solutions of NaCl or KCl at different ionic strength, there were no significant differences between DSC scans in water and 500 mM salt.

Curve d in Figure 5 was obtained by sonication of DPPG in 100 mM NaCl buffer, and subsequent concentration by reduced pressure ultrafiltration. The ultrafiltration was necessitated by the need to concentrate the sonicated suspension to 1/5 the original volume in order to obtain a sample concentrated enough for the DSC. When this sample was left overnight at 45° the peak had become sharper with midpoint at 41°, resembling more closely the unsonicated suspension. Such change did not occur when the sample was incubated for a similar period of time at 24°. These changes are similar to those reported by Prestegard and Fellmuth (1974) for dimyristoyllecithin vesicles.

The presence of bivalent metals has a pronounced effect on the thermotropic properties of the acidic lipids, but again no significant effect on DPPC. The addition of Mg^{2+} (10 mM final concentration) to a sonicated dispersion of DPPG at room temperature produces visible flocculation. The pellet after brief centrifugation gives the result shown as curve e (solid line) in Figure 5. The transition is now much sharper than sonicated dispersions in NaCl (Curve d) and 12.5° higher than the main transition of the unsonicated DPPG (curve b). Repeated heating and cooling of the same sample (broken and dotted lines under curve e) results in transitions of decreasing enthalpy. In another experiment, a small, broad peak centering at 50° was obatined after incubation of dry DPPG for 1 hr at 45° in 100 mM NaCl (pH 7.4) containing 1 mM $MgCl_2$. The value of the enthalpy for that transition was less than 1/10 of that reported for DPPG in NaCl solution (Table II).

These effects of Mg^{2+} should be contrasted to the results obtained with Ca^{2+}. Thus in experiments similar to the one shown above in curve e, Figure 5, the addition of $CaCl_2$ (10 mM) resulted also in floculation, but the sample showed no transition at all between 20 and 70°. Titration of DPPG samples with increasing concentration of $CaCl_2$ indicated a definite shift of the peak to higher temperatures before the total disappearance of the transition. Complete loss of the peak was obtained when Ca^{2+} was added at an equimolar ratio to DPPG. The presence of either Ca^{2+} or Mg^{2+} at concentrations of 1–3 mM in 100 mM NaCl (pH 6.0) buffer during the suspension of DPPA results in total absence of an endothermic peak between 20 and 87°.

The effect of bivalent metals was also investigated with phosphatidylserine, in order to obtain correlations with previous work (Papahadjopoulos and Bangham, 1966; Papahadjopoulos, 1968). Figure 6 shows the DSC scan of a dis-

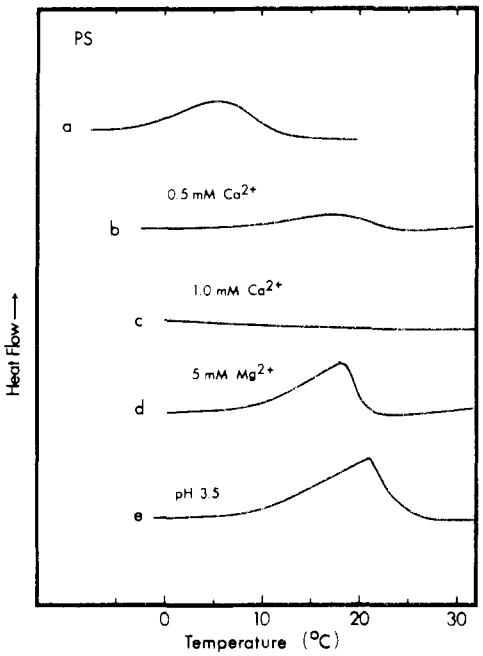

FIGURE 6: Differential scanning calorimetry for PS. Heating rate, 5°/min. All dispersions prepared in 100 mM NaCl. Thermograms (a–c) for PS at pH 7.4 and $CaCl_2$ concentrations of 0.0, 0.5, and 1.0 mM, respectively. No transition was apparent up to 80°; (d) thermogram for PS in 5 mM $MgCl_2$ at pH 7.4; (e) thermogram for PS at pH 3.5. Details of sample preparation, as under Materials and Methods.

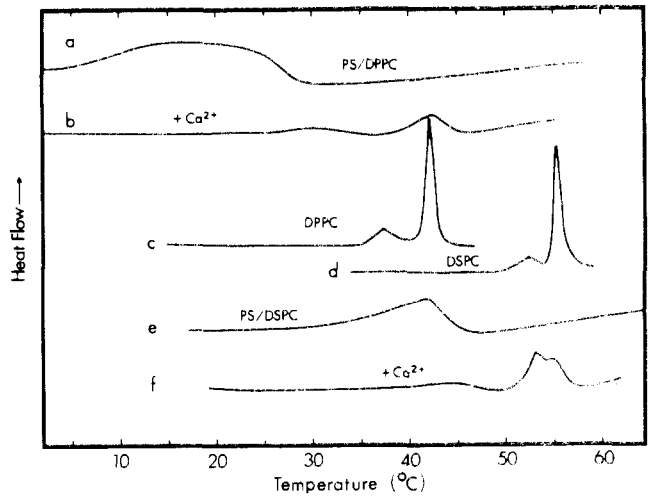

FIGURE 7: Differential scanning calorimetry thermogram on mixed lipid membranes before and after addition of Ca^{2+}. Phospholipid dispersions were made in 100 mM NaCl buffer (pH 7.4) at concentrations of 4 μmol/ml. (a) PS/DPPC (2:1) mixture, dispersed without sonication for 30 min at 37°; (b) PS/DPPC (2:1) mixture, dispersed without sonication for 30 min at 37°, then incubated for 30 min with Ca^{2+} (10 mM) at 37°; (c) pure DPPC nonsonicated dispersions dispersed at 42°; (d) pure DSPC, nonsonicated, dispersed at 58°; (e) PS/DSPC (2:1) mixture dispersed without sonication at 42°; (f) PS/DSPC (2:1) mixture, dispersed by sonication for 1 hr at 42° then incubated for 1 hr with Ca^{2+} (10 mM) at 42°. If EDTA (equimolar to Ca^{2+}) is added after the above Ca^{2+} treatment, the calorimeter scan of the sample is identical with that shown in (e). All the above samples were centrifuged at room temperature for 10 min at 100,000*g* and the pellets transferred into the calorimeter sample pans with a pasteur pipet. Amount of phospholipid per sample was approximately 1 μmol.

persion of beef brain phosphatidylserine suspended in 100 mM NaCl at pH 7.4. A broad endothermic peak centers around 5° (curve a). If the above experiment is performed in the presence of 0.5 mM $CaCl_2$ (curve b), the endothermic transition is shifted to higher temperatures with a broader peak now centering at 18°. This peak becomes broader and shallower at 0.7 mM $CaCl_2$, and disappears completely when $CaCl_2$ concentration is raised to 1.0 mM (curve c). This concentration of $CaCl_2$ was shown earlier to be necessary for an increase in permeability of PS vesicles (Papahadjopoulos and Bangham, 1966) and PS bilayers (Papahadjopoulos and Ohki, 1969).

Absence of a definite phase transition is also observed when sonicated vesicles of PS (in 100 mM NaCl, pH 7.4) are dialyzed against the same buffer containing 1.0 mM $CaCl_2$. In this case a precipitate is formed, which was shown earlier to contain PS and Ca^{2+} at a molar ratio of 2:1 (Papahadjopoulos and Bangham, 1966) and to consist of well-packed lamellae (Papahadjopoulos and Miller, 1967). Recent X-ray diffraction studies with PS membranes suspended in 100 mM NaCl containing 3 mM $CaCl_2$ have shown a sharp 4.2-Å spacing suggestive of crystalline paraffin chain packing, as well as a sharp 4.5-Å spacing, whose origin is not understood at the present (S. Hui, personal communication) As shown in curve d, Figure 6, dialysis of the same suspension of PS vesicles against 100 mM NaCl at pH 7.4 containing 5mM $MgCl_2$ results in a precipitate which exhibits an endothermic transition centering at 18°. Differences between Ca^{2+} and Mg^{2+} in their ability to increase the permeability of PS vesicles and to condense PS monolayers were reported earlier (Papahadjopoulos, 1968).

The endothermic transition of PS can also be shifted to higher temperatures when it is suspended at low pH. As shown in Figure 6, curve e, the transition centers at 20° when this phospholipid is suspended at pH 3.5. At this pH a substantial proportion of the carboxyl groups of the serine are protonated, and thus the PS molecule would be internally neutralized (Papahadjopoulos, 1968). When the pH of the suspending medium is 2.5, the transition centers at 26°, with an enthalpy similar to that of PS at pH 7.4 (ΔH = 4.5 ± 0.5 kcal/mol). This represents a 15–20° shift to higher temperatures, following the neutralization of a negative charge. This result is in contrast to that of Träuble and Eibl (1974) who report a slight decrease in T_c with DPPS as pH is decreased from 7 to 2.

Phase Separation in Mixed Lipid Vesicles Induced by Ca^{2+}. Work recently reported from this laboratory has presented evidence for molecular segregation (phase separation) in mixed lipid vesicles induced by Ca^{2+} (Papahadjopoulos *et al.*, 1974). Phase separation has been shown to occur in mixed lipid systems with widely different melting points (Phillips *et al.*, 1972) such as dioleoylphosphatidylcholine and distearoylphosphatidylcholine (DSPC). We have attempted to induce such phase separation, in lipid mixtures that do not separate normally but give a single broad transition. Two such systems are described in Figure 7.

Curve a in Figure 7 was obtained with a mixture of 66% PS and 34% DPPC suspended in 100 mM NaCl at pH 7.4. A very broad peak is observed between 5 and 28°. When the same mixture was suspended in the same buffer, but in the presence of 10 mM $CaCl_2$, two peaks were obtained (curve b): one low peak centering at 29° and another more pronounced centering at 42°. This result indicates a phase separation of "pure" DPPC, induced by the binding of Ca^{2+} to PS. Curve c shows the behavior of pure DPPC with a main peak centering also at 42°. The small peak at 29° probably represents the melting of PS–Ca^{2+} domains which still contain considerable amounts of DPPC, since pure PS

with Ca^{2+} shows a peak at 18° or no peak at all, depending on Ca^{2+} concentration. Similar results are obtained when $CaCl_2$ is added at 10 mM concentration to a sonicated dispersion of 66% PS: 34% DPPC. In this case the more pronounced peak is at 28°, with a second peak also at 42°.

In order to substantiate the above conclusions concerning phase separation of pure DPPC, the experiments were repeated with a mixture consisting of 66% PS in DSPC. The calorimetric behavior of this mixture is shown in curve e, where a single broad peak is centered at 42°. Curve f was obtained from such a mixture after sonication and addition of $CaCl_2$ (10 mM). The new double peak appearing at 53 and 55° must be a new phase containing mostly DSPC since this lipid alone shows a main peak at 55.5° (curve d). The effect of Ca^{2+} was found to be reversible. Following addition of EDTA (20 mM) to a sample such as that shown in curve f, one obtains a curve similar to curve e.

It should be noted here that the addition of Mg^{2+} (up to 20 mM) to such PS/DSPC mixtures fails to induce a similar phase separation. Similarly, a decrease of the pH of the buffer from 7.4 to 3.5, or addition of cytochrome *c*, or polylysine did not induce phase separation with mixtures of 66% PS in DPPC. It can thus be concluded that calcium ions are quite specific in inducing phase separation in such mixtures. As noted earlier (Papahadjopoulos *et al.*, 1974), Ca^{2+} does not induce phase separation below a characteristic concentration and, then, only with mixtures containing more than 50% proportion of PS in PC.

Mixtures of 66% DPPA with 34% DPPC were also studied for phase separation. These mixtures give a single broad peak centering at a temperature of 64° at pH 6.5 and 55° at pH 8.5. When the same mixtures are suspended in the presence of 3 mM $CaCl_2$, the suspension at pH 6.0 shows no transition up to 70°. On the other hand, the suspension at pH 8.0 (3 mM Ca^{2+}) shows a broad peak centering at 42°, indicating the separation of DPPC domains. As mentioned above with PS/DPPC mixtures, no phase separation was achieved by suspending the DPPA-DPPC mixture at pH 2.9. The thermogram obtained with such a mixture shows a single transition at 69° with no new peak in the region of 42°.

Discussion

Use of Fluorescent Probes to Detect Lipid Bilayer Phase Transitions. Since appreciable discrepancies between the fluorescence probe perylene and DSC methods of phase transition detection do not occur with either DPPC or DPPG, it is of interest to consider the origin of the difference with DPPA, especially because the probe DPH reports accurately the DPPA transition at pH 6.5. DSC data at pH 6.5 is not affected by the presence of perylene at 1:500 (Per/DPPA) ratio, indicating that the small mole fraction of probe present does not change the T_c. As mentioned in the Results section, either polarization or intensity may be misleading indicators of fluidity, while rotation rate should be the most unambiguous criterion. However, calculations of probe rotation rate *vs.* temperature do not reveal appreciably altered transition onset and end points when compared to polarization and intensity scans. We conclude the discrepancy is real and must reveal something about perylene's relationship to the DPPA bilayer structure.

As discussed later, one effect of ionizing DPPA may be to produce a more "defect" structure in the gel state caused by intermolecular charge repulsion. Perylene, which is probably excluded from the acyl chain crystal lattice at temperatures well below the T_c (Papahadjopoulos *et al.*, 1973a), could move into this postulated "defect" structure prior to the onset of lattice melting from a position in the interfacial region. This is recorded by the enhanced probe fluorescence and decreased polarization before any appreciable excess specific heat is recorded by DSC. Since both fluorescence parameters show the transition to be completed before the thermogram indicates completion, the probes may be segregated in liquid-like "domains" as the transition progresses. The appearance of these domains may be enhanced by the presence of the probe.

One intriguing aspect of this comparative study is the result obtained with DPH. The transition with DPPA dispersions at pH 6.5 is reported in exact agreement with the DSC results. This rod-like molecule exhibits almost limiting polarizations below the T_c ($p_0 = 0.46$ (Shinitzky and Barenholz, 1974) and p(DPPA) = 0.445 at 25°). This indicates that DPH can "cocrystallize" with DPPA acyl chains below the T_c. Melting of the bilayer causes the largest drop in polarization yet observed ($\Delta p = 0.30$) in bilayer phase transitions. This supports the contention of Shinitzky and Barenholz (1974) that DPH is a sensitive fluorescent probe for bilayer fluidity.

The behavior of DPH and perylene can be contrasted. The postulated mobility of perylene is probably due to its disc-like molecular shape which is not readily accommodated by crystalline bilayer lattices. Thus, its average position in the bilayer will be a function of the physical state of the membrane which complicates the interpretation of the fluorescence data. On the other hand, the interpretation of DPH results is simplified because it is truly a hydrocarbon region probe to the extent that it will even cocrystallize with bilayers undergoing a phase transition.

Effect of Head Group on the Phase Transition of Dipalmitoyl Lipid Membranes. The data summarized in Table II show that the chemical nature and charge of the lipid head group can clearly affect melting point, but not always in a manner based on simple electrostatic considerations. A prime example, as reported several years ago, is dimyristoylphosphatidylethanolamine which, when dispersed in water, melts at 48°, 25° higher than dimyristoylphosphatidylcholine (Ladbrooke and Chapman, 1969; Oldfield and Chapman, 1972), even though both are neutral. From the present study, the similarity of melting parameters for DPPC and DPPG is striking since DPPG carries one negative charge at pH 7.4. In contrast, even doubly ionized DPPA melts about 16° *higher* than DPPC. However, in comparing DPPA at pH values above and below the pK_2, $DPPA^{2-}$ would be expected to melt at lower temperatures than $DPPA^-$ and in fact it was observed to melt 9° below $DPPA^-$. The theory adopted by Träuble and Eibl (1974) may not quantitatively predict this change in T_c because it cannot completely account for the observed changes in ΔH and ΔS induced by increasing surface charge. Specifically, the electrostatic effects treated in this theory account for only a small part (0.4 kcal/mol) of the measured transition enthalpy differences between DPPC and $DPPA^-$ or between $DPPA^-$ and $DPPA^{2-}$ (2.5 and 2.3 kcal/mol, respectively). Finally, large decreases in the transition entropy were observed (Table II) and the theory assumes this quantity to be independent of surface charge.

To provide a qualitative framework for understanding these melting data we consider, in accord with Nagle (private communication), the transition enthalpy to consist of the sum of terms arising from gauche isomer formation, ex-

pansion against the van der Waals forces, disruption or alteration of interfacial and head-group structures (such as intermolecular linkages, possible chelation by multivalent cations, structured water, etc), and the difference in the electrical double layer energies ($\Delta\Gamma$) as approximated by Träuble and Eibl (1974, eq 1 and 2). Each term represents the difference between the energy associated with the disordered and the ordered state. All these terms should be considered in understanding the bilayer melting behavior. Specifically, ionization of head groups to produce charged bilayers will not, in general, affect merely the electrical double layer but may also produce structural alterations in the hydrocarbon region particularly important in the gel state. These alterations will affect ΔH and ΔS, both of which must be considered in predicting the transition temperature (*i.e.*, $T_c = \Delta H/\Delta S$ for a first-order transition). All the above considerations assume a unique gel to liquid-crystalline transition. However, it should be acknowledged that differences in the head group region could result in gel phases of different crystallographic parameters. Such phase differences would also have to be considered in any detailed comparison of the thermotropic properties of a given family of phospholipids.

Despite the present lack of complete crystallographic data on phospholipids other than lecithin (Tardieu *et al.*, 1973), some possible explanations for the diverse melting behavior of phospholipids differing in head groups can be offered. The higher melting point of PE compared to homologous PC could be rationalized in terms of intermolecular ion pair formation between phosphate and amine (Papahadjopoulos and Weiss, 1969; Michaleson *et al.*, 1974). An analogous explanation could be advanced for DPPA, involving hydrogen bonds between vicinal phosphates. Such lateral attractive interactions could result in the diminished values of ΔH and ΔS primarily due to increased constraint of acyl chain motions in the fluid state. On the other hand, in the absence of compensating lateral interactions, the repulsive forces between ionized phosphate groups could have a disordering effect, particularly in the gel state. This effect would also result in decreased ΔH and ΔS with concomitant effects on T_c. Such reasoning could also apply to DPPA especially in the doubly ionized form. Additional data from techniques such as dilatometry and nuclear magnetic resonance would be helpful in assessing the contribution of various lateral interactions to the thermotropic properties of lipid bilayers.

Effects of Ionic Environment on Phase Transitions and Separations in Vesicle Membranes. Changes in monovalent ion concentrations, bivalent metal concentration, or pH have diverse effects on the properties of pure acidic bilayers (such as PG, PA, or PS) and mixed acidic–neutral bilayers. Similar changes have no appreciable effect on the properties of the electrically neutral PC bilayers.

Increasing ionic strength (NaCl) has a small but definite effect in increasing the T_c of DPPG. Qualitatively, such an effect is expected on the basis of increased screening of the negative charge as NaCl concentration is increased. This result is not in agreement with the conclusions of Träuble and Eibl (1974) that monovalent cations "fluidize" acidic phospholipid bilayers. However, these conclusions were based on the behavior of PA at pH values near the pK_2, and the "fluidizing" effect was acknowledged as being due to a shift in the ionization of the PA^- to PA^{2-}. Such ionization is not possible with the PG where the phosphate is a diester, and thus the effect of increasing ionic strength is observed only as slightly increasing the stability of the bilayer.

Addition of Ca^{2+} and Mg^{2+} can produce an increase in the T_c of DPPG and PS to an extent dependent on the ion species and concentration. This is in agreement with earlier observations (Träuble and Eibl, 1974; Kimelberg and Papahadjopoulos, 1974; Verkleij *et al.*, 1974) and indicates a remarkable stabilization of the bilayers by low concentration of bivalent metals. As reported in a previous paper (Papahadjopoulos *et al.*, 1973b) when the concentration of Ca^{2+} is higher than 1 mM, the phase transition of PS is completely absent within the temperature range tested (0–70°). This occurs also with DPPG at a molar ratio DPPG/Ca of 1.0. The appearance of a sharp 4.2-Å spacing in the X-ray diffraction pattern of PS bilayers at 25° in the presence of 3 mM Ca^{2+} indicates the existence of a crystalline state. The occurrence of a transition at much higher temperatures is indicated by the results of Verkleij *et al.* (1974) in a comparable system. We postulate that lower concentrations of Ca^{2+} gradually increase the transition temperature by charge neutralization. However, as the Ca^{2+} (bound)/PS or DPPG approaches $\frac{1}{2}$, a phase transition occurs, which is somewhat analogous to that induced by cooling through the T_c. The effects of Ca^{2+} and Mg^{2+} are distinctly different[3] with either PS or PG. This difference cannot be rationalized in total by postulating an electrostatic effect (lipid charge neutralization within the Stern layer) by the binding of Ca^{2+} and Mg^{2+} to acidic phospholipid membranes (McLaughlin *et al.*, 1971; Träuble and Eibl, 1974). Specific binding of Ca^{2+} to PS with the formation of polymeric complexes was postulated earlier (Papahadjopoulos, 1968).

Phase transitions can be induced isothermally also by changes in pH as shown by the results given in Figure 4 with DPPA bilayers. This effect is most pronounced near the temperature of the phase transition. Our results confirm those of Träuble and Eibl (1974) on DMPA.

Addition of Ca^{2+} to mixed lipid bilayers can induce lateral phase separation isothermally, with the neutral PC molecules segregating from the acidic PS or DPPA molecules. This is in agreement with earlier observations on spin-labels and mixtures of PS in PC (Ohnishi and Ito, 1973; 1974). The conditions under which Ca^{2+} induces phase separation are similar to those found to be necessary for fusion between phospholipid bilayers (Papahadjopoulos *et al.*, 1974). Thermally induced phase separations in lipid mixtures containing species of different melting points have been reported by Phillips *et al.* (1972) and also by Shimshick and McConnell (1973). The ability of phospholipids to segregate into domains is of considerable physiological interest, especially in relation to the possible existence of microenvironments in the vicinity of membrane proteins. The ability of Ca^{2+} to segregate clusters (or domains) of acidic phospholipids does not appear to be due to simple charge neutralization. As mentioned earlier in the Results section, neutralization of PS by either a decrease in the pH or by addition of Mg^{2+}, does not produce any detectable phase separation, although these agents tend to increase the T_c of the lipid mixtures. In addition, phase separation was observed with DPPA/DPPC mixtures only at pH 8.0 and not at pH 6.0, indicating a requirement for two negative charges per molecule. This would favor the formation of lin-

[3] Träuble and Eibl (1974) reported that Ca^{2+} and Mg^{2+} had qualitatively the same effects on PA, in general agreement with earlier findings with PA monolayers (Papahadjopoulos, 1968).

ear polymers with Ca^{2+} as postulated earlier for PS and PA (Papahadjopoulos, 1968).

Isothermally induced phase separations, such as those described above, could play an important role in modulating enzyme activity and other functions in biomembranes. For example, consider the following possibilities for the involvement of Ca^{2+} in the regulation of cation transport. A carrier-like transport peptide has an affinity for acidic lipid domains. The carrier is inactive when Ca^{2+} binding to the lipid induces crystallinity in the domain as shown for valinomycin conductance in thermally induced bilayer phase transitions (Krasne *et al.*, 1971). Upon Ca^{2+} release, however, the domain becomes "fluid" and the carrier conducts. Alternatively, consider an asymmetric membrane domain having acidic lipids on one side only (Bretcher, 1973; Zwaal *et al.*, 1973). Binding of Ca^{2+} to the acidic lipid side prevents complete penetration of channel (or carrier) through the membrane and thereby blocks transport. Release of Ca^{2+} allows complete transmembrane penetration of channel or carrier so that the site conducts. Another possibility is that the components of a pore are disordered in an acidic lipid domain when it is fluid, but upon addition of Ca^{2+}, the pore components cocrystallize with the acidic domain to form conductive channels.

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References

Bangham, A. D., Standish, M. M., and Watkins, J. C., (1965), *J. Mol. Biol. 13,* 238.

Bretcher, M. (1973), *Science 181,* 622.

Chapman, D., Urbina, J., and Keough, K. M. (1974), *J. Biol. Chem. 249,* 2512.

Chen, R. F. (1967), *Anal. Biochem. 20,* 339.

Cogan, U., Shinitzky, M., Weber, G., and Nishida, T. (1973), *Biochemistry 12,* 521.

Dawson, R. M. C. (1967), *Biochem. J. 102,* 205.

Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T., and Wakil, S., (1971), *Proc. Nat. Acad. Sci. U.S. 68,* 3180.

Faucon, J. F., and Lussan, C. (1973), *Biochim. Biophys. Acta 307,* 459.

Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.

Jacobson, K., and Wobschall, D. (1974), *Chem. Phys. Lipids 12,* 117.

Kimelberg, H. K., and Papahadjopoulos, D. (1972), *Biochim. Biophys. Acta 282,* 277.

Kimelberg, H. K., and Papahadjopoulos, D. (1974), *J. Biol. Chem. 249,* 1071.

Krasne, S., Eisenman, G., and Szabo, G. (1971), *Science 174,* 412.

Ladbrooke, C. D., and Chapman, D. (1969), *Chem. Phys. Lipids 3,* 304.

Lanyi, J. (1974), *Biochim. Biophys. Acta 356,* 245.

McLaughlin, S. G. A., Szabo, G., and Eisenman, G. (1971), *J. Gen. Physiol. 58,* 667.

Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1974), *Biochemistry 13,* 2605.

Nagle, J. F. (1973a), *J. Chem. Phys. 58,* 252.

Nagle, J. F. (1973b), *Proc. Nat. Acad. Sci. U.S. 70,* 3443.

Nicholls, P., and Wenner, C. E. (1972), *Arch. Biochem. Biophys. 151,* 206.

Ohnishi, S.-I., and Ito, T. (1973), *Biochem. Biophys. Res. Commun. 51,* 132.

Ohnishi, S.-I., and Ito, T. (1974), *Biochemistry 13,* 881.

Oldfield, E., and Chapman, D. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 23,* 285.

Overath, P., Schairer, H. V., and Stoffel, W. (1970), *Proc. Nat. Acad. Sci. U.S. 67,* 606.

Papahadjopoulos, D. (1968), *Biochim. Biophys. Acta 163,* 240.

Papahadjopoulos, D. (1970), *Biochim. Biophys. Acta 211,* 407.

Papahadjopoulos, D., and Bangham, A. D. (1966), *Biochim. Biophys. Acta 126,* 185.

Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T. (1973a), *Biochim. Biophys. Acta 311,* 330.

Papahadjopoulos, D., and Miller, N. (1967), *Biochim. Biophys. Acta 135,* 624.

Papahadjopoulos, D., and Ohki, S. (1969), *Science 164,* 1075.

Papahadjopoulos, D., and Ohki, S. (1970), *in* Liquid Crystals and Ordered Fluids, Johnson, J. F., and Porter, R. S., Ed., New York, N.Y., Plenum Press, p 13.

Papahadjopoulos, D., Poste, G., and Shaeffer, B. E. (1973b), *Biochim. Biophys. Acta 323,* 23.

Papahadjopoulos, D., Poste, G., Schaeffer, B. E., and Vail, W. J. (1974), *Biochim. Biophys. Acta 352,* 10.

Papahadjopoulos, D., and Weiss, L. (1969), *Biochim. Biophys. Acta 183,* 417.

Phillips, M. C., Hauser, H., and Paltauf, F. (1972), *Chem. Phys. Lipids 8,* 127.

Phillips, M. C., Williams, R. M., and Chapman, D. (1969), *Chem. Phys. Lipids 3,* 234.

Prestegard, J. H., and Fellmuth, B. (1974), *Biochemistry 13,* 1122.

Raison, J. K. (1973), *Bioenergetics 4,* 285.

Robles, E. C., and Van Den Berg, D. (1969), *Biochim. Biophys. Acta 187,* 520.

Sackmann, E., Träuble, H., Galla, H.-J., and Overath, P. (1973), *Biochemistry 12,* 5360.

Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11,* 4573.

Shimschick, E., and McConnell, H. M. (1973), *Biochemistry 12,* 2351.

Shinitzky, M., and Barenholz, Y. (1974), *J. Biol. Chem. 249,* 2652.

Tardieu, A., Luzzati, V., and Reman, F. C. (1973), *J. Mol. Biol. 75,* 711.

Träuble, H., and Eibl, H. (1974), *Proc. Nat. Acad. Sci. U.S. 71,* 214.

Träuble, H., and Overath, P. (1973), *Biochim. Biophys. Acta 307,* 491.

Verkleij, A. J., DeKruyff, B., Ververgaert, P. H. J. Th., Tocanne, J. F., and Van Deenen, L. L. M. (1974), *Biochim. Biophys. Acta 339,* 432.

Wilson, G., Rose, S. P., and Fox, C. F. (1970), *Biochem. Biophys. Res. Commun. 38,* 617.

Yi, P. N., and MacDonald, R. C. (1973), *Chem. Phys. Lipids 11,* 114.

Zwaal, R. F. A., Reolofsen, B., and Colley, C. M. (1973), *Biochim. Biophys. Acta 300,* 159.