Ionic Influences on the Phase Transition of Dipalmitoylphosphatidylserine†

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ABSTRACT: The ionization and phase behavior of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine have been investigated under a variety of conditions by several different methods. As measured by turbidity changes, the temperature of the crystal–liquid crystal phase transition of this lipid is influenced by pH and mono- and divalent cation concentrations. The pH–transition temperature curve is congruent with the curve relating temperature to the degree of ionization of the carboxyl group of the crystalline form. The transition temperature falls from an upper plateau of 72 °C at low pH values, where the carboxyl group is fully protonated, to a lower plateau of 55 °C at high pH values, where this group is fully ionized. The apparent pK (pH at 50% ionization) of the crystalline form shifts from 6.0 to 4.6 to 3.7 with an increase of NaCl concentration from 10^{-3} to 0.1 to 1.0 M, respectively. These observations are in accord with a simple theoretical analysis that utilizes diffuse double layer theory and the influence of surface potential on surface concentration of protons. In qualitative terms, an increase in electrolyte concentration reduces the surface potential, the result of which is a diminution of the surface–bulk pH difference and a lowering of the apparent pK. Assuming an area of 50 Å^2/molecule, the intrinsic pK_a (apparent pK corrected for surface pH) of the carboxyl group is 2.7. A 1000-fold change of NaCl concentration produces a very large change in surface potential without influencing the transition temperature of the ionized form of the lipid. This observation, coupled with the correspondence of the transition temperature change with the change of degree of ionization, suggests that intermolecular hydrogen bonding, and not electrostatic forces, determines the way transition temperature changes with pH. The enthalpy of the lipid phase transition falls from about 9 kcal/mol to about 3 kcal/mol as the protonated lipid is converted to the ionized form. Titrations of the crystal and liquid crystal phases reveal that the apparent pK of the former is one unit higher than that of the latter; about 30% of this difference is accounted for by the lower surface charge density of the latter (larger area per molecule) and the remainder is due to a lower intrinsic pK. As a result of the smaller affinity of the liquid crystal phase for protons, the phase transition (melting) is accompanied by a release of protons that is detectable by conductivity, electrophoretic, and pH measurements. An increase in electrophoretic mobility is associated with melting even at high pH values and cannot be attributed to additional ionization of protons but is probably due to hydrodynamic effects. Calcium and magnesium ions bind strongly to phosphatidylserine vesicles, increasing the transition temperature and removing lipid from the phase melting below 72 °C. As a result of diminished proton competition, the interaction of divalent cations is stronger the higher the pH. Sodium and potassium ions reduce interaction with divalent cations by depressing the surface potential and reducing the surface concentration of the divalent ion.

It is currently believed that a major structural feature of most biological membranes is a lipid bilayer, which, depending upon temperature, is in a more or less viscous fluid state. Upon cooling under some conditions, membrane lipids undergo a phase transition into an ordered, two-dimensional crystalline state (Steim et al., 1969; Engelman, 1971).[1] Since many membrane enzymes and transport proteins must function within the bilayer matrix, the possibility exists that the state of membrane lipids could exert some control over membrane function. It was therefore of considerable interest that membrane transport was indeed found to be influenced by membrane lipid phase transitions (Overath et al., 1970). Temperature-dependent effects are not likely to be important for homoisothermic organisms, so if lipid phase transitions do play a role in cellular membrane function, they would have to be triggered isothermally. Isothermal transitions are indeed possible in lipids bearing a net charge, as has been demonstrated by others (Träuble and Eibl, 1974; Verkleij et al., 1974; Jacobson and Papahadjopoulos, 1975). In these cases, changes in either pH or salt concentration may provoke the transition.

We have investigated the physical and electrochemical basis of ionic influences on the crystal–liquid crystal phase transition of dipalmitoylphosphatidylserine. A serine phosphatide was chosen because these compounds are usually the most abundant acidic lipids in animal cell membranes and the palmitic acid derivative was chosen with the expectation that it would exhibit a sharp transition. We found that the phase transition temperature can be changed by about 20 °C by changes in the ionic composition of the aqueous phase. The influence of the aqueous solution on phase stability is explicable on the basis of elementary electrochemistry in most cases.

Materials and Methods

Dipalmitoylphosphatidylserine (DPPS)[2] was prepared

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[1] The use of the word "crystal" in the present context does not necessarily imply that lipids at low temperatures possess all of the properties (e.g., ability to support shear stress) normally attributed to crystals.

[2] The following abbreviations are used: DPPS, dipalmitoylphosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; T_e, endpoint transition temperature.

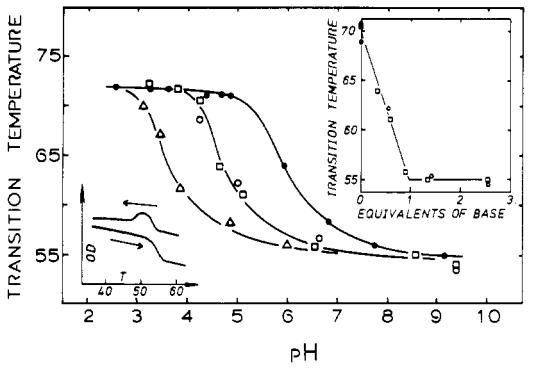

FIGURE 1: pH and electrolyte concentration dependence of DPPS transition temperature. DPPS phase transition end point temperatures are plotted as a function of the pH of the lipid dispersion at 25 °C. Lipid concentrations varied from 0.25 to 1.0 mg/ml. Aqueous phases were: (△) 1.0 M NaCl + 0.05 M phosphate buffer; (□) 0.1 M NaCl; (○) 0.1 M KCl; (●) no added electrolyte. In the latter three cases sufficient NaOH or KOH was present in the aqueous phase so that the pH values of the dispersions were as shown on the abscissa. pH values were determined at 25 °C after the transition temperature measurements. Inset, upper right: Transition temperature of DPPS as a function of the degree of neutralization. Data of 0.1 M NaCl (□) and 0.1 M KCl (○) series of main part of the figure are plotted against the equivalents of base that had been added to the aqueous phase prior to dispersing the lipid in it. Inset, lower left: Optical density (ordinate) of DPPS dispersion as a function of temperature (abscissa). DPPS was dispersed in distilled water containing sufficient NaOH that the pH (at 25 °C) was 7.7, and the degree of ionization about 0.9. Lipid concentration was 1 mg/ml. Lower and upper curves are heating and cooling curves, respectively.

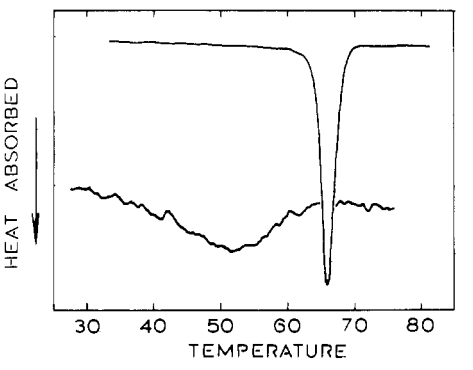

FIGURE 2: Differential scanning calorimetry of DPPS. These scans were obtained on a DuPont Model 990 equipped with a differential scanning calorimeter sample chamber. Heating rate was 2 °C/min. Upper curve: DPPS in water at pH 3.2 (at 23 °C). Lower curve: DPPS in water containing slightly more than 1 equiv of NaOH. Noise in the lower curve is due to a lower attenuation setting.

according to the procedure of Baer and Maurukas (1955). Water was distilled, passed through charcoal and ion exchange cartridges, and redistilled. Salts were reagent grade and, to mitigate possible organic contamination, occasionally roasted or extracted with chloroform.

Lipid dispersions for turbidity measurements were prepared by injecting a 25 mg/ml solution of DPPS in warm tetrahydrofuran into a stirred aqueous solution of the appropriate composition. In most cases volumes were chosen to give dispersions containing 0.25–1 mg of lipid per ml of aqueous phase. The tetrahydrofuran was removed by evaporating the dispersion to about one-half of its initial volume on a rotary evaporator and then adding water to restore the original volume. The tetrahydrofuran was purified over an activated alumina column before use. This method of dispersion was preferred to sonication because the latter method, for this lipid, required prolonged sonication at elevated temperatures. A similar method, when applied to dipalmitoylphosphatidylcholine, yields a population of single-layered vesicles (Batzri and Korn, 1973). However, sonication was used to produce coarse dispersions for electrophoresis or to disperse preparations made by the solvent injection method which had subsequently aggregated.

Optical density measurements were done on a Perkin-Elmer Model 124 spectrophotometer. The sample was contained in a water-jacketed cell and its temperature varied by heating or cooling the water circulated through the jacket. The heating rate was 3–4 °C/min and the cooling rate about 5 °C/min. A linear thermistor circuit with the probe inserted directly into the sample permitted plotting temperature vs. optical density on an x–y recorder. Accuracy and precision of the temperature measurements were about ±0.5 °C.

Conductivity was measured at 1000 Hz with voltages of between 100 mV and 1 V, using an AC ammeter and platinum black electrodes.

Microelectrophoresis was done with an apparatus like that described by Bangham et al. (1958).

Calorimetric measurements were made both with a Perkin-Elmer DSC-1B and on a DuPont 990. Sealable sample pans were used with lipid samples of around 1 mg. Bulk DPPS does not hydrate rapidly until it is heated to 90 °C (the phase transition temperature of the anhydrous lipid), so the samples were either equilibrated in an oven or the first scan was carried to this temperature. Thereafter, scans were repeated twice or until reproducible results were obtained. The instruments were calibrated frequently with appropriate pure metals or organic compounds.

Hydrogen ion titrations were done on dispersions of 5–10 mg of lipid in about 5 ml of degassed 0.1 M NaCl solution. Aliquots of standardized 0.1 M HCl or NaOH were added from a microliter syringe. Identical volumes of NaCl solutions without lipid were titrated to permit correction for the buffer capacity of water.

Results

pH Dependence of the Phase Transition Temperature. The pH dependence of the transition temperature of DPPS, as determined by turbidity measurements, is illustrated in Figure 1. The temperatures shown in this and in subsequent figures of this kind represent the temperature at the end of the transition (T_e).[2] This point was chosen because of its rather sharp cut-off as compared to the more gradual onset of the transition. Data are presented in Figure 1 for several different electrolyte concentrations. In each instance, the transition temperature shifts from 55 to 72 °C as the pH is decreased. The pH range over which this shift occurs, however, is higher, the lower the electrolyte concentration.

The relationship between DPPS ionization and its phase transition temperature was obtained by plotting pH against the number of equivalents of base used in the 0.1 M NaCl and KCl samples of Figure 1. Such a plot is given in the inset of that figure (upper right), from which it is obvious that the transition temperature change is a linear function of the degree of ionization.

In univalent electrolytes the optical density–temperature heating scans that were used to establish the T_e's for the data such as those of Figure 1 corresponded to those of DPPC (Abramson, 1971), and are taken to likewise result from the lipid becoming less dense and less ordered upon

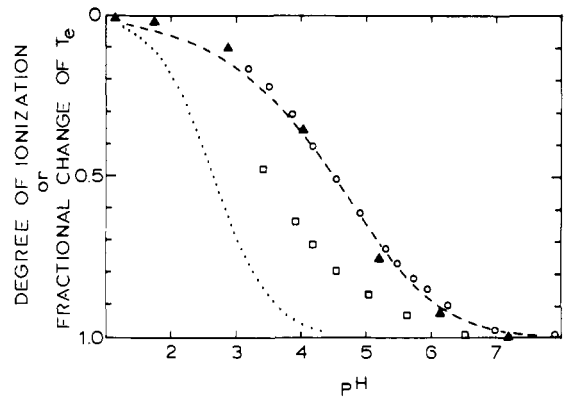

FIGURE 3: pH dependence of T_e and degree of ionization. Degrees of ionization at 25 °C of crystalline DPPS (○) and at 75 °C of liquid-crystalline DPPS (□) as obtained from hydrogen ion titrations are plotted against bulk pH of dispersion. pH dependence of T_e (▲, as fractional change) corresponds to pH dependence of degree of ionization of crystalline but not to that of liquid-crystalline DPPS. Dashed line is bulk pH dependence of degree of ionization calculated from eq 1-3 (see Discussion) with $pK_a = 2.7$. Dotted line is surface pH dependence of degree of ionization calculated from eq 1 with $pK_a = 2.7$. Abscissa is bulk pH for all except dotted line in which case it is surface pH.

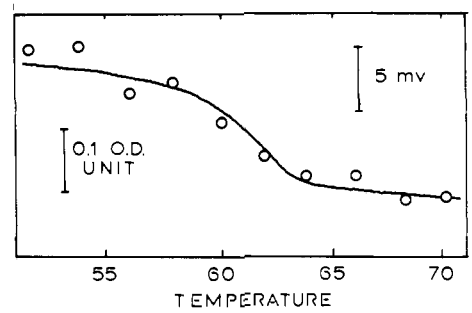

FIGURE 4: Comparison of temperature dependence of optical density and ζ potential of DPPS dispersion. A coarse dispersion of DPPS was prepared by sonicating 2 mg of DPPS in 10 ml of 0.1 M NaCl + 0.01 M Na_3PO_4-H_3PO_4 buffer. The optical density as a function of temperature was determined on 1 ml of the sample. The remaining 9 ml was subjected to the same thermal history as the 1 ml. The two portions were combined and the ζ potential of the DPPS determined as a function of temperature. The pH of the sample was 3.8 after cooling to 25 °C. Circles represent ζ potential and the line represents optical density.

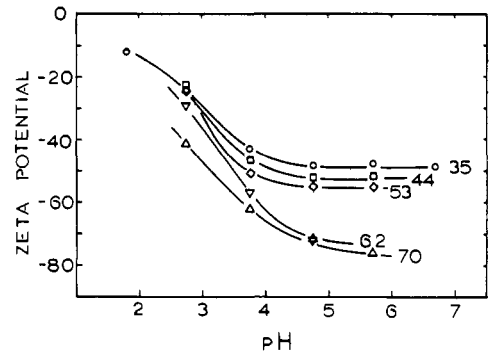

FIGURE 5: pH and temperature dependence of ζ potential of DPPS. DPPS was dispersed in 0.1 M NaCl containing 0.01 M Na_3PO_4 and 0.01 M H_3PO_4 in various proportions. Lipid concentration was 0.2 mg/ml. ζ potentials were determined at the temperatures shown at the ends of the curves. The pH (at 25 °C) of the samples after electrophoresis was as shown on the abscissa.

melting (Yi and MacDonald, 1973). At pH values above about 6.5 the optical density-*cooling* curves of DPPS differed from those of DPPC in that the former, instead of retracing the heating curves, exhibited a hump just under the transition temperature. This behavior is illustrated in the inset in the lower left of Figure 1.

Calorimetry confirms that an increase in pH lowers the phase transition temperature and also reveals that the transition enthalpy is much lower for the ionized than for the protonated lipid. Calorimeter scans of DPPS in water, in which the DPPS is almost completely protonated and of DPPS in NaOH solution in which it is almost completely ionized, are presented in Figure 2. The protonated form melts sharply at 69 °C with an enthalpy of about 9.0 kcal/mol, whereas the ionized form exhibits a much broader transition centered at about 53 °C with an enthalpy of only about 3 kcal/mol. Since the lipid concentrations are very much greater (~100 times) than those used in the optical experiments, no significance can be attached to the small difference in transition temperatures measured by the two methods.

To characterize a possible difference between the pK of DPPS above and below the transition temperature, the lipid was titrated at 25 and at 75 °C. Degree of ionization-pH relationships derived therefrom are presented in Figure 3. The apparent pK (pH at 50% ionization) of the liquid-crystalline form is about 1 unit below that of the crystalline form. The degree of ionization-pH curve of the latter corresponds to the transition temperature (normalized to the range 0-1)-pH curve. Outside the transition, the temperature dependence of the pH of these samples is so small that the fact that the titrations were not done just above and just below the transition temperature makes little difference. The dotted and dashed lines of Figure 3 are explained in the Discussion.

The relationship between transition temperature and pH is based upon simple equilibration of protons between DPPS and the aqueous phase; pH extremes have no irreversible effects on DPPS samples within the time necessary to do these experiments. When acid and base were added alternatively to lipid samples, the transition temperature cycled between the upper and lower extremes, respectively.

Other Correlates of the Lipid Phase Transition. A pH decrease corresponding to a drop in pK of about 0.8 unit and a marked increase in conductance (arising from increased proton dissociation) were observed to coincide with the optical changes that mark the phase transition.

A direct measure of the change in electrophoretic mobility that accompanies the phase transition was obtained by microelectrophoresis. Figure 4 depicts a comparison between the ζ potential of a DPPS dispersion and the optical density of the sample, both as a function of temperature. The ζ potential becomes more negative (increased proton dissociation) at the same temperature at which the optical density decreases. This experiment was done at pH 3.8, so, according to Figure 3, the degree of dissociation increased from about 0.3 to 0.6 during melting.

Additional electrophoretic measurements over a wider pH range revealed that the change in ζ potential can be larger than expected from the known change of degree of dissociation that accompanies the phase transition of DPPS. In Figure 5 are plotted ζ potentials as a function of pH at several different temperatures. These data reveal the expected change of ζ potential to a more negative value, corresponding with an increased dissociation of protons, in the regions of pH where DPPS is largely protonated (see

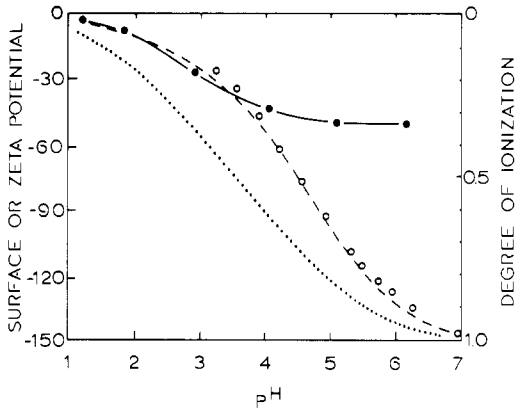

FIGURE 6: Dependence of surface and ζ potential upon pH: (●) measured ζ potential plotted vs. pH of DPPS dispersion (0.1 M NaCl plus 0.01 M phosphate buffer). Dotted curve is pH dependence of surface potential calculated from eq 2 and 3 with $pK_a = 2.7$. Dashed curve and O are calculated and observed degrees of ionization from Figure 3. ζ potential differs markedly from degree of ionization and from calculated surface potential.

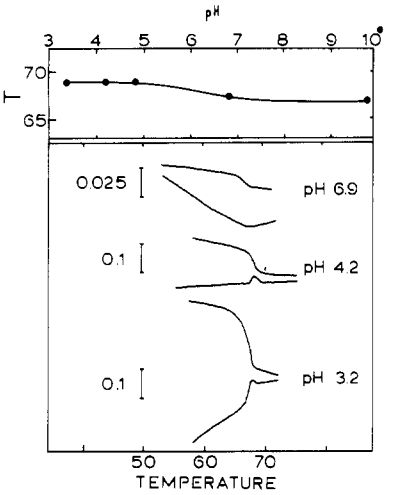

FIGURE 7: Phase transition of DPPS in $MgCl_2$ solutions. Procedures were as described in the legend for Figure 1 except that the aqueous phase was 10^{-3} M $MgCl_2$. Upper part of the figure shows pH dependence of the transition temperature; lower part of the figure shows the temperature dependence (first heating and cooling) of the optical density of three of the samples represented in the upper part. The lower of each pair of curves is the heating run and the upper of each pair is the cooling run. Bars represent optical density units as labeled.

change from 62 to 70 °C between pH 2.5 and 3.5). They also reveal, however, a very substantial change of ζ potential at pH values above 6 in the temperature range of the transition (compare 53 °C with 62 °C). This change is clearly larger than that predicted from the temperature dependence of the ζ potential (including bulk viscosity and dielectric constant changes) and since, according to the ionization curves of Figure 3, the crystalline form is already about 95% ionized above pH 6.5, such a change must reflect an increase in electrophoretic mobility that is independent of the surface charge density increase, i.e., a change in some other electrostatic parameter or in a hydrodynamic parameter.

It should be recognized that the pH values of the half-maximal values of the ζ potentials of Figure 5 are more than 1 pH unit lower than the pH at half-ionization of Figure 3. A direct comparison of the ζ potential–pH (filled circles) and the degree of ionization–pH (open circles) curves is presented in Figure 6. Also included in the figure is the theoretical curve from Figure 3 (dashed line) and the ζ potential–pH relationship calculated therefrom (dotted line). These theoretical curves are considered in the Discussion, but it may be mentioned here that the disagreement between calculated and observed ζ potentials, as opposed to the agreement between calculated and observed degree of ionization, is probably due to the familiar underestimation of surface potential by ζ potential measurements of highly charged surfaces in moderately concentrated electrolyte solutions.

Effects of Divalent Cations on the Properties of DPPS Dispersions. In contrast to the effect of monovalent cations, the chlorides of calcium and magnesium affect both the transition temperature at the ionization limits and the magnitude of the optical density change accompanying the transition. Magnesium chloride at 10^{-3} M raises the transition temperature at high pH values by more than 10 °C (Figure 7, upper part). In the lower part of Figure 7 are shown three of the optical scans used to construct the curve in the upper part. For the moment, attention is directed to the cooling scans (upper curve of each pair). These reveal a larger optical density change for transitions at low pH values than at high. This reflects a stronger interaction of magnesium ion the more highly charged the lipid, i.e., protons and the divalent cation compete for the carboxyl group. As will be seen below, when the ratio of divalent cation to hydrogen ion concentration is sufficiently high, the transition is completely eliminated, at least at these temperatures.

In addition to these effects, divalent cations influence the shape of the optical density–temperature scans on the first heating (Figure 7, lower curve of each pair) differently, depending upon the pH. This behavior contrasts with that of the cooling curves and of the reheating curves, both of which consist only of two plateau regions separated by a smooth transition. These effects are of significance since they clearly indicate that the crystalline phase presents a barrier to interaction with the cation that is circumvented by cycling the system through the liquid-crystalline form. Other acidic lipids also exhibit two low-temperature phases, i.e., one which involves a metastable interaction with divalent cations and another which is a stable state but which forms rapidly only when the lipid passes through its phase transition in the presence of the divalent ion (Ververgaert, et al., 1975).

When the experiment of Figure 7 was repeated with calcium chloride instead of magnesium chloride, the lipid dispersion no longer exhibited a transition that could be detected by the optical method unless the pH was below 5. Similarly, at high pH values when calcium ion concentration was comparable to lipid concentration, transitions were not seen anywhere near the expected temperatures by scanning calorimetry.

Titrations of DPPS with $CaCl_2$ at pH 7 revealed that the transition temperature rose from 56 to 69 °C as the Ca/lipid ratio approached 1:2. A similar increase in T_e was seen with $MgCl_2$ but required about three times as much magnesium as calcium ion. In the presence of KCl the effectiveness of both divalent ions was reduced, as would be expected from the double layer effects considered in the Discussion. The influence of divalent ions on DPPS appears to be much the same as those of ions on dilauroylphosphatidylglycerol reported by Verkleij et al. (1974) where, with small increases in the ratio of divalent ion to lipid, small increases

in transition temperature are seen, but then when the ratio exceeds 1:2 the transition rather abruptly jumps to a temperature about 50 °C higher. This phase melting at high temperatures evidently corresponds to the "cochleate" cylinders observed by Papahadjopoulos et al. (1975) when brain phosphatidylserine was treated with calcium ion.

Association of calcium ion with DPPS, like proton association, is a simple equilibration, and no irreversible changes ensue; the increase of the transition temperature upon addition of calcium ion is reversed upon chelation of that ion with EGTA. Thus, pH, univalent, and divalent cations are all capable of triggering isothermal transitions of DPPS.

Discussion

Phase Transition of DPPS in Solutions of Univalent Electrolytes. As was shown by Figures 1 and 3, the change of the transition temperature of DPPS follows the degree of ionization. The degree of ionization as a function of pH can be analyzed in terms of an intrinsic ionization constant:

$$K_a = ([H_s^+][PS^-])/[HPS] \quad (1)$$

where K_a is the constant and the terms in brackets represent concentrations of hydrogen ions (at the lipid surface) and of ionized and protonated DPPS. (For simplicity, activity coefficients will be taken to be unity.) In addition, cognizance must be taken of the influence of the surface potential on the surface-bulk distribution of protons. To a good approximation, the surface potential (φ_0) will be related to the bulk electrolyte concentration (C) and the surface charge density (σ) according to the Gouy-Chapman equation:

$$\varphi_0 = (2RT/F) \sin h^{-1}(500\pi/DRTC)^{1/2}\sigma \quad (2)$$

where D is the aqueous phase dielectric constant and R, T, and F have their usual meanings. Protons will distribute themselves between surface and bulk phases according to the Boltzmann equation:

$$[H_s^+]/[H_b^+] = e^{-\varphi_0 F/RT} \quad (3)$$

where $[H_b^+]$ represents the bulk concentration of hydrogen ions. In addition, we have pH = $-\log [H_b^+]$. Since the surface potential becomes more negative, the greater the degree of ionization, the surface concentration of protons becomes larger relative to the bulk concentration, the higher the pH.

Equations 1-3 were solved simultaneously to give $[PS^-]/[HPS]$ as a function of pH. The charge density was taken as 100 000 esu/cm^2, corresponding to about 50 Å^2 per DPPS molecule. This is close to the area of the similar lipid, dipalmitoylphosphatidylcholine, below its transition temperature (Chapman et al., 1967). With pK_a = $-\log K_a$ = 2.7, the calculated curve (dashed line of Figure 3) corresponds quite well with the degree of ionization curve that was derived from hydrogen ion titrations, as well as with the transition temperature-pH curve. The pK_a defined by K_a of eq 1 is not commonly determined. It is a measure of the strength of interaction of a proton with a single phosphatidylserine molecule in the absence of surface electrostatic effects. What is commonly measured is the apparent pK, which is considered below.

Träuble and Eibl (1974) have observed with fluorescence techniques that the phase transition temperature of dimyristoylphosphatidic acid also decreases by about 20 °C in proportion to the degree of ionization of the second proton. These investigators have also determined the pH dependence of the transition temperature of DPPS and report a relationship quite different both from what we find and from what they found for phosphatidic acid; they remark only that the behavior of their DPPS is not easily explained. Possibly the fluorescent probe is responsible.

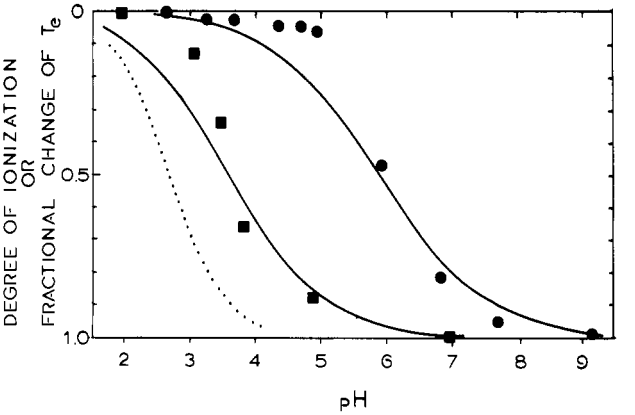

FIGURE 8: Comparison of pH dependence of temperature and degree of ionization. Fractional change of temperature plotted vs. pH for DPPS in 1.0 M NaCl (■) and in water with appropriate NaOH to adjust pH (●). Lines are pH dependencies of the degree of ionization for NaCl concentrations corresponding to those of the experimental data and were calculated from eq 1-3 with pK_a = 2.7. Abscissa is bulk pH for all but dotted line which is the same as that of Figure 3 for which the abscissa is surface pH.

Effects of Monovalent Electrolytes. Since the surface potential depends upon the electrolyte concentration according to eq 2, the proton concentration at the vesicle surface will differ from that in the bulk aqueous phase. This is the familiar bulk-surface pH differential that is to be expected for any charged surface. The transition temperature-pH curves move to the right (higher pH) with decreasing salt concentration (Figure 1). Such a shift is predicted from eq 1-3.

Figure 8 presents a comparison of the calculated degree of ionization curves with transition temperature curves (normalized to the range 0 to 1) for the dispersions in 1.0 M NaCl (■) and in no added salt (●) from Figure 1. The assumption in comparing these curves is that the intrinsic pK_a is invariant at 2.7 and that the transition temperature follows the degree of ionization as it did in 0.1 M electrolyte (Figure 3). The point of half-ionization corresponds to the theoretical curves, but there is some discrepancy between the data and the calculated curves at low degrees of ionization. This could be due to an underestimation of the upper limit of the phase transition temperature. If, for example, the true value were 74 °C at infinite proton concentration, these points would fall very close to the theoretical curves. In the case of the calculation for the dispersion made in the absence of added salt, C of eq 3 was approximated by the cation concentration. Such an approximation introduces a significant error only when the surface potential is very small, which it was not in these experiments.

Although the *surface* pH at which the DPPS vesicle surface is 50% ionized corresponds to the intrinsic pK_a and is independent of salt concentration, the *bulk* pH at which the surface is 50% ionized varies considerably with electrolyte concentration. The latter pH is usually referred to as the apparent pK. The apparent pK we obtained for the crystalline phase (25 °C) was 4.6 and that of the liquid crystalline phase (75 °C) was 3.5, both by titration in 0.1 M NaCl. The pH values of half-maximal transition temperature

change in 1.0 M NaCl and in 10^{-3} M NaCl (case of no added salt; sodium ion concentration taken for the point of half-neutralization) were 3.7 and 6.0, respectively (Figure 8). By analogy with the situation for 0.1 M salt, these pH values should be very nearly the same as the corresponding apparent pK values.

To illustrate the substantial effect of surface charge on the surface–bulk pH differential, we have included, in Figures 3 and 8 (dotted line), the solution of eq 1 with pK_a = 2.7. This curve represents the degree of ionization of DPPS as a function of surface pH. For a given degree of ionization, the shift to higher pH from the dotted curve to the full curves of Figure 8 and to the dashed curve of Figure 3 represents the difference between the surface and bulk pH for the corresponding salt concentration. It should be noted that in 0.1 M salt (Figure 3), this difference is appreciable even at rather low degrees of ionization.

Measurements of the apparent pK of *brain* phosphatidylserine in the literature (Hendrickson and Fullington, 1965; Abramson et al., 1964; Papahadjopoulos, 1968; Seimiya and Ohki, 1973) give apparent pK values between about 3.2 and 4.5; all are in agreement that these apply to the ionization of the serine carboxyl group.

Since the transition temperature–pH curve is translated along the pH axis by a change in salt concentration, a change of salt concentration at constant pH and temperature can cause a phase change. Träuble and Eibl (1974) have reported a salt concentration dependent phase change of phosphatidic acid. They likewise ascribe it to a change in degree of ionization resulting from the effect of salt concentration on surface potential and, thereby, on surface pH. Jacobson and Papahadjopoulos (1975) have also reported that the transition temperature of phosphatidic acid is influenced by salt concentrations at pH values where the lipid is partially ionized.

Mechanism of Influence of Degree of Ionization on Transition Temperature. Träuble and Eibl (1974) have suggested that the linear change with degree of ionization of the transition temperature of phosphatidic acid may be ascribed to differential electrostatic repulsion in the crystalline and liquid-crystalline phases. The latter is more expanded than the former, so that melting would reduce the repulsion energy. They argue that the reduction of repulsion with melting should increase with ionization; hence melting should occur at lower temperatures, the greater the degree of ionization. There are several difficulties with this hypothesis. Träuble and Eibl (1974) have calculated that for phosphatidic acid, the transition temperature should change by about 20 °C with the conversion of the monoprotonated to the fully ionized form, a value in good agreement with their measured value and, incidentally, with the corresponding change for DPPS measured by us. One problem is that their calculation requires the assumption that the transition entropy be independent of the degree of ionization. This is not true, as shown by Jacobson and Papahadjopoulos (1975) for phosphatidic acid and by us for DPPS. Jacobson and Papahadjopoulos also point out that electrostatic repulsion cannot account for the transition enthalpy difference between the states of ionization of phosphatidic acid. We have found an even larger difference with DPPS. An additional objection to electrostatic repulsion as the factor controlling the transition temperature is the constancy of the lower limit of the transition temperature (fully ionized lipid) with salt concentration changes. The change of surface potential upon changing the salt concentration from 1 to 10^{-3} M is far greater than the change in potential that could occur by expansion of the membrane at the phase transition (at any of these salt concentrations), so if the latter potential change accounted for the 20 °C shift of T_e with pH, the former should cause the T_e of ionized DPPS to change by much more than 20 °C over the range of salt concentration tested. In fact, T_e of ionized DPPS was independent of salt concentration (Figure 1).

The present evidence indicates that a weak bond, involving the carboxyl proton, is formed between adjacent molecules in the crystalline phase but not—perhaps because of increased intermolecular separation—in the liquid-crystalline phase. It appears likely that this is a hydrogen bond to the carbonyl or unsubstituted phosphate oxygens on adjacent molecules, but it is also possible that the carboxyl group shares a proton with an adjacent amino group. Such suggestions of hydrogen bonding among polar groups of phospholipids are by no means new, having been suggested, in various forms, by Abramson et al. (1965), Papahadjopoulos and Weiss (1969), and Gitler (1971). This explanation is consistent with the pK reduction observed upon melting of the crystalline phase, with the absence of an effect of salt concentration on the transition temperature of fully ionized DPPS, and since bonding to water would be diminished, also with the extreme hydrophobicity of the crystalline phase of protonated DPPS (Ladbrooke and Chapman, 1969).

The 6-kcal difference in phase transition enthalpy between the two phases is probably too large to be attributed only to breaking a hydrogen bond; however, the polar head groups of the liquid crystal should, because of their larger area of contact with the aqueous phase, be more hydrated than those of the crystal and such hydration could easily account for several kilocalories.

Phase Transition Dependent Changes Surface Charge Density. Although an increase of ζ potential corresponds to the phase transition at fixed pH values (Figure 4), the ζ potential–pH relationship found corresponds very poorly with the degree of ionization–pH relationship (compare filled circles with open circles of Figure 6). This is to be expected for a highly charged surface, where a significant fraction of the counterions reside within the shear layer (Davies and Rideal, 1963; Aveyard and Haydon, 1973). In addition, some of the assumptions used in the derivation of eq 2 also become questionable at high charge densities.

In general, the pH at half-maximal surface potential will be found considerably lower than the pH at which the surface is 50% ionized (compare dotted and dashed lines of Figure 6). This difference follows from the fact that surface potential is not a linear function of the degree of ionization (eq 2). Thus, contrary to what has sometimes been assumed, the surface potential and especially the ζ potential *cannot* be used to establish either an apparent or an intrinsic pK without additional calculations based on eq 1–3. That the pH at which the ζ potential is half-maximal (Figure 6) is close to the pK_a obtained by hydrogen ion titration is merely fortuitous.

It was unexpected that the ζ potential would become appreciably more negative at the transition temperature of dispersions that were almost fully ionized. A pK_a decrease can account for such a change only if there are un-ionized carboxyl groups available for dissociation; this was not true at several of the pH values where an increase in electrophoretic mobility was seen. For example, in 0.1 M NaCl at pH 5.75 and 53 °C, the ζ potential of DPPS was −55 mV; at

the same pH but at 70 °C, the ζ potential was −75 mV (Figure 5). In the former case, the degree of ionization is about 0.9 and in the latter about 0.95. This is much too small a change of degree of ionization to account for the change of ζ potential. Under such conditions, then, the change of ζ potential must depend upon electrokinetic factors other than surface charge density. Of such factors, viscosity at the shear layer appears most susceptible to influence by the state of the lipid, although other parameters that perhaps could change concomitant with the phase transition are surface dielectric constant, particle shape, and extension of the carboxyl group from the bilayer surface.

Influence of Divalent Cations. Increasing concentrations of salts of calcium and magnesium cause a shift of the transition of DPPS to higher temperatures. This effect has been reported by Jacobson and Papahadjopoulos (1975) with brain phosphatidylserine and by Träuble and Eibl (1974) with DPPS.

The loss of a phase transition, at least in the normal temperature range, in the presence of large amounts of divalent cations is apparently due to the appearances of new structures with a much higher transition temperature involving strong bilayer–bilayer adhesion (Verkleij et al., 1974; Papahadjopoulos et al., 1975).

The calcium ion titrations suggest that, at least for calcium ion, the binding stoichiometry is 1 Ca/2 DPPS. This ratio is commonly found for the interaction of natural acidic lipids with divalent cations (Dawson and Hauser, 1970). Such a ratio implies that the ion bridges a pair of lipid molecules (see Papahadjopoulos, 1968) and, we believe, accounts for the increase in the DPPS transition temperature. Träuble and Eibl (1974), on the other hand, believe that divalent ion binding raises the transition temperature by lessening electrostatic repulsion preferentially in the crystalline phase. While it is true that the effects of the ions are greater, the greater the degree of ionization, it is also true that ionization favors stronger binding. (The distinction between electrostatic screening and chemical binding is discussed by McLaughlin et al., 1971.) Since concentrated univalent cations, which affect intermolecular electrostatic repulsion indirectly, have no influence on the transition temperature of the ionized lipid, it appears to us unlikely that the effect of divalent ions is predominantly electrostatic.

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