Electrostatic Effects on Lipid Phase Transitions: Membrane Structure and Ionic Environment

(Triggering factors/cation binding/pH-dependence/ionic strength/double layer energy)

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Communicated by Manfred Eigen, September 11, 1973

ABSTRACT Ordered → fluid phase transitions in bilayers of charged lipids are accompanied by a decrease in electrostatic free energy mainly as a result of bilayer expansion. For uniform charge distribution the Gouy–Chapman theory of the electrical double layer predicts a decrease of the transition temperature with increasing charge density. We studied the effects of pH and monovalent and divalent cations on the phase transition of lecithin, cephalin, phosphatidylserine, and phosphatidic acid bilayers. Phosphatidic acid with two ionizable protons was selected for a systematic investigation. A change in pH from 7 to 9 increases the charge per polar group from one to two elementary charges. This lowers the transition temperature by about 20°C in agreement with the theory. In this pH region rather small changes in pH suffice to induce the phase transition at constant temperature. Divalent cations (Mg2+ and Ca2+) increase the transition temperature by charge neutralization and thus can be used to induce the fluid → ordered transition at constant temperature. In contrast, monovalent cations (Li+, Na+, K+) lower the transition temperature, and fluidize the bilayer structure at a given temperature. Rather small changes in ionic environment can induce gross alterations in bilayer structure; divalent and monovalent cations have antagonistic effects. This result parallels current theories on nerve excitation and sensory transduction where cation-induced structural changes in biomembranes are invoked.

Thermal phase transitions in lipid bilayers and intact membranes have attained considerable interest (review in ref. 1). With increasing temperature the following conformational changes occur at the critical temperature, Tc: (a) formation of rotational isomers within the lipid hydrocarbon chains (2–4), (b) the onset of rapid lateral diffusion of the lipid molecules (5, 6) (ordered → fluid transition), and (c) a considerable expansion of the bilayer area (7, 8). For dipalmitoyl-lecithin (C16-Lec) (Tc = 41°C), the molecular area, f, increases from 48 Å² (T < Tc) to 60–70 Å² (T > Tc).

Temperature has been used to trigger lipid phase transitions. Biological systems, however, are remarkably constant in temperature; hence conformational changes in vivo must be caused by variables other than temperature. We decided to test whether lipid phase transitions can be triggered at constant temperature by monovalent and divalent cations and by changes in pH. Such effects are to be expected for charged lipid bilayers because the electrostatic free energy, Γ, of the system, changes at Tc as a result of bilayer expansion. Therefore, all parameters affecting the value of Γ can be expected to alter the transition temperature. In a certain temperature range these variables may thus be used to induce the phase transition at a constant temperature.

Theory

For a reversible phase transition at constant pressure the molar free energies of the two states are equal at the critical temperature, Tc. Therefore ΔH = Tc ΔS, where ΔS denotes the entropy change and ΔH the enthalpy change, or the heat absorbed at the ordered → fluid phase transition. ΔH may be written as a sum of a nonelectrostatic term, ∆H°, and an electrostatic term ∆F = Γ(fluid) – Γ(ordered). The change in Tc caused by electrostatic effects is given by ΔTc = Tc – Tc° = ΔF/ΔS, where Tc is the observed transition temperature and Tc° = ∆H°/ΔS is the value of Tc in the absence of electrostatic interactions.

If the membrane charges are assumed to be uniformly distributed over the membrane surface, ΔF can be expressed in terms of the difference in the electrical double layer energy, φ, of the two states. φ may be calculated on the basis of the Gouy–Chapman theory (9). For a 1:1 electrolyte and high surface potentials* the free energy of the interface per cm² is

\[ \phi = 2(kT/e)(\sigma'/f) \]  

independent of the electrolyte concentration. Half of this value is obtained for 2:1 electrolytes. In this expression k, T, and e have their usual meaning; f is the area of one lipid molecule and \( \sigma' \) is the charge per polar group (specific charge). For phosphatidic acid (PA), \( \sigma' \) is connected with the degree of dissociation, α, of the phosphate groups by \( \phi = e \cdot \alpha \). \( \sigma'/f \) is equivalent to the surface charge density, α.

Using Eq. 1 and denoting by ∆f = f2 – f1 the increase in molecular area at the ordered → fluid phase transition, one calculates† for the change in electrostatic free energy per mol

\[ \Delta \phi = 2(kT/e)(\sigma'/f) \]

* This expression is correct to within 2% (10%) for \( \sigma' > e \) (0.5 e) and \( f \approx 60 \text{Å}^2 \) and for electrolyte concentrations smaller than 0.5 M.

† It is assumed that the specific charge, \( \sigma' \), is the same below and above Tc. For PA bilayers this is not strictly true. The bulk pH of unbuffered PA dispersions (2 mM) decreases abruptly by ΔpH < 0.3 pH units when the samples are heated above Tc. Maxima of ΔpH are observed at pH 4 and 9. The necessary corrections to ΔF are, however, negligibly small.
\[ \Delta G = -2RT \left( \frac{\Delta f}{f_1} - \frac{\Delta f}{f_2} \right) \frac{\sigma^\prime}{e} \]  

Here \( \sigma^\prime = \sigma + f_1 \) and \( f_2 \) are the molecular areas for \( T < T_c \) and \( T > T_c \), and \( R \) denotes the gas constant (1.987 cal/deg mol). Hence, the corresponding change in \( T_c \) is

\[ \frac{\Delta T}{\Delta S} = -\gamma \sigma, \text{ with } \gamma = \frac{2RT}{\Delta S} \left( \frac{\Delta f}{f_1} - \frac{\Delta f}{f_2} \right). \]  

Thus, for highly charged bilayers \( T_c \) is expected to decrease linearly with increasing surface charge \( (\sigma^\prime = \sigma + \alpha) \) or increasing \( \alpha \). The physical picture of the charge-induced phase transition is quite simple. The electrostatic free energy of the fluid bilayer is smaller than in the ordered state (\( \Delta G \) is negative) and therefore an increase in surface charge favors the fluid state.

Values of \( \Delta S \) have been measured (10) for lecithins of different chain length. For example, \( \Delta S = 22.4 \text{ cal/deg mol for } \) C14-Lec. Molecular areas have been determined for C16-Lec as \( f_1 = 48 \text{ Å}^2 (T < T_c) \) and \( f_2 = 70 \text{ Å}^2 (60 \text{ Å}^2) \) for \( T > T_c \), where 70 Å\(^2\) is the value at full hydration and 60 Å\(^2\) at 20\% hydration (7, 8). Using these values we may estimate \( \Delta T \) from Eq. 3. Considering a change in \( \sigma^\prime \) from \( e \) to \( 2e \) at \( T = 320\text{K} \), one obtains \( \Delta T = -18^\circ \text{C} \) (12) for \( f_2 = 70 \text{ Å}^2 \) (60 Å\(^2\)). The corresponding value of \( \Delta T \) is 400 cal/mol compared with \( \Delta H^\circ = 6.64 \text{ kcal/mol for } \) C14-Lec (10).

In the experiments \( \sigma \) can be varied (a) by changing the pH or (b) by the adsorption of cations to negatively charged lipids. In (a) \( T_c \) is expected to decrease with increasing pH, whereas in (b) \( T_c \) increases. The ionic strength is not included explicitly in Eq. 3.

A systematic study of these effects was made with dispersions of PA. PA was chosen because its structure is relatively simple and because it has two ionizable protons. PA may exist as \( \text{H}_2\text{PA}, \text{HPA}^-, \text{and PA}^- \) (see Fig. 3). Thus, the specific charge, \( \sigma' \), can be varied between zero and 2 \( e \) by changing the pH. In addition, the assumption of a uniform charge density should be a valid approximation.

The effect of the pH on \( T_c \) was studied also for C14-Lec, C16-Lec, C14-Ceph, and dipalmitylphosphatidylserine (PS) with more complex polar groups.

**Materials and methods**

PA was prepared from C14-Lec by enzymatic degradation with phospholipase D. C14-Lec and C14-Ceph were products of Fluka. PS was purchased from Serdary (Canada) and purified by chromatography. Divalent cations were removed by EDTA.

The lipids were dispersed ultrasonically at temperatures \( T > T_c \) and diluted to \( 2.5 \times 10^{-4} \text{ M} \). Phase transitions were indicated using the uncharged, polarity-dependent fluorescence probe \( N\text{-phenyl-naphthylamine} \) (purchased from Kodak) in final concentrations of about 1 \( \mu \text{M} \). NPN reports the ordered fluid phase transition by a large increase in fluorescence intensity (Fig. 1) mainly as a result of a higher partitioning of the dye into the hydrocarbon phase of fluid bilayers (11).

\[ \text{Fig. 1. Fluorescence indication of lipid phase transition using NPN as indicator. PA dispersion: } 2.5 \times 10^{-4} \text{ M lipid, } 1.7 \times 10^{-4} \text{ M NPN, pH 6. Excitation: } 350 \text{ nm; emission: } 420 \text{ nm. The value of } T_c \text{ was determined as the temperature where } \theta = 0.5. \text{ The maximal rate of temperature change was } 1^\circ \text{C/min} (11). \]

\[ \text{does not influence the phase transition for molar ratios of NPN/lipid smaller than } 10^{-2} (31). \]

In a few cases 90\(^\circ\)-light scattering was used to indicate lipid phase transitions (12).

**Results and discussion**

**pH Dependence of the Transition Temperature.** Fig. 2 shows the pH dependence of \( T_c \) for different lipids. \( T_c \) is sensitive to variations in pH in those regions where the ionization of the polar groups is affected. This is the region below pH 3 for lecithin and cephalin (phosphate groups) and the region above pH 9 for cephalin (amino group). PA has sensitive regions below pH 4.5 and above pH 7 (Fig. 2b). The behavior of PS is determined by its three ionizable protons. For appropriate values of pH and temperature, lipid phase transitions can be triggered by rather small changes in pH. For example, for PA (0.5 M NaCl) at 40\(^\circ\)C a variation in pH between pH 7.5 and 8.5 suffices to induce the phase transition in either direction. This was confirmed by pH titrations.

The results for PA may be relatively easily explained. Here an increase in pH releases a first proton between pH 1 and 3 and a second proton between pH 7.5 and 10 (Fig. 3) resulting in a stepwise increase of \( \sigma \) from 0 to 2\( e \). In qualitative agreement with Eq. 3, this leads to a stepwise decrease of \( T_c \). This decrease is paralleled by an increase in the width of the transition; for example at pH 3.5, 6.0, and 10 the width of the transition is about 2.5\(^\circ\)C, 3.5\(^\circ\)C, and 7.5\(^\circ\)C, respectively. A quantitative comparison with theory may be made by plotting the values of \( T_c \) against the degree of dissociation. A fairly straight line is obtained with a slope \( dT_c/d\alpha = - (23 \pm 1)\text{°C} \) (Fig. 2c) close to the theoretical value \( dT_c/d\alpha = -18\text{°C} \) estimated from Eq. 3). For this estimate we have used values of \( \Delta S, f_1, \) and \( f_2 \) valid for C14-Lec (\( \Delta S \)) and C16-Lec \( (f_1, f_2) \) in the hope that they approximate the unknown values for PA.

The results for C14-Lec and C14-Ceph and for PS are less easily explained. The findings for cephalin and PS (at high pH) fulfill the criterion (valid for PA) that an increase in \( \sigma' \) tends to lower the value of \( T_c \). However, lecithin exhibits just the opposite behavior. In these cases the electrostatic

\[ \frac{\Delta G}{\Delta T} = -2RT \left( \frac{\Delta f}{f_1} - \frac{\Delta f}{f_2} \right) \frac{\sigma^\prime}{e} \]  

\[ \text{However, for small surface potentials } \phi \text{ is proportional to } \sigma'/\sqrt{n} \text{ (n = 1:1 electrolyte concentration). Therefore, } T_c \text{ is expected to increase with increasing } n \text{, as inferred from the screening effect of counterions in the diffuse double layer.} \]
interaction takes place between extended polar groups which may carry positive and negative charges simultaneously. An estimate of the electrostatic free energy for these systems requires assumptions as to the orientation of the polar groups.

Divalent Cations. The reaction of divalent cations with negatively charged lipids is critically important for many biological processes (13, 14). Specific adsorption of divalent cations to negatively charged bilayers will reduce the surface charge and thus, according to Eq. 3, increase $T_t$. We studied the effects of $Mg^{++}$ and $Ca^{++}$ on the transition of PA between pH 6 and 10. All these cations, in rather small concentrations, strongly increase $T_t$ (Fig. 4). A saturation value of $T_t$ is approached for molar ratios $m = [Me^{++}]/[PA] \approx 0.5$. Qualitatively the same effects are produced by $Ca^{++}$ and $Be^{++}$. In addition, divalent cations broaden the transition and produce hysteresis. All these effects may be reversed by adding EDTA.

The effects of divalent cations, judged by the initial slope $(dT_t/dm)_{m=0}$ and the net increase in $T_t$, increase with increasing pH or increasing concentration of $PA^-$ (Fig. 4, inset). This result suggests that the divalent cations interact preferentially with $PA^-$. In fact, several authors (15-18) have proposed that the process $Me^{++} + PA^- \rightleftharpoons MePA$ is the major reaction between $Me^{++}$ and $PA^-$. However, the residual effect of divalent cations observed at lower pH (inset, Fig. 4) indicates that binding to HPA$^-$ also occurs. Qualitatively the same behavior is observed for PS at high pH. For example,

at pH 9.5 addition of $Mg^{++}$ to a molar ratio $[Mg^{++}]/[PS] \approx 0.5$ increases $T_t$ by about 8°C.

These results imply that divalent cations for properly chosen values of pH and temperature can induce the fluid → ordered transition in bilayers of PA and PS at a constant temperature. This confirms the proposed role of divalent cations as membrane “stabilizers” (13).

Other “charge-reducing” compounds are effective in a similar way. For example, addition of polylysine [poly(Lys)] to dispersions of PA at pH 8.5 increases $T_t$ by several degrees centigrade, indicating that the $NH_3^+$-groups of poly(Lys) are in close contact with the phosphate groups of PA.

Monovalent Cations. Monovalent cations do not (or only

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$\S$ The experiments can be explained by the assumption that the polar groups of lecithin are oriented almost perpendicular to the bilayer surface, whereas in cephalin they are nearly parallel to the bilayer surface.

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Fig. 2. Effect of the pH on the transition temperature of different phospholipids. Unbuffered dispersions containing $2.5 \times 10^{-4}$ M lipid and $2 \times 10^{-3}$ M NPN. (a) C14-Lec, C16-Lec (pK $\geq 1.95$), and C14-Ceph. Measurements at decreasing ($\times,$ $\times$) and increasing ($\Delta,$ $\Delta$) temperature. (b) PA and PS. Vertical bars indicate the range of $T_t$ measured at increasing and decreasing temperature. (c) Transition temperature of PA (0.5 M NaCl) against the degree of dissociation, $\alpha_2$ for pH 6.5-11. The values of $T_t$ are taken from b, the values of $\alpha_2$ from Fig. 3 (PA, 0.5 M NaCl).

Fig. 3. Dissociation of the phosphate groups in PA bilayers and in the soluble glycerol phosphate; derived from acid-base titrations (PA and GP: $3 \times 10^{-3}$ M). The higher pK of PA is due to electrostatic forces attracting the protons to the negatively charged bilayer surface.
static forces attracting a constant $K_0$, takes a form given by reaction (1), which is an equation with a constant (see $\alpha_2$).

This pK equation is valid for monovalent and divalent cations, lowering the surface potential $\psi_0$ for a highly-charged surface and a 1:1 electrolyte. The relevant equation is

$$\psi_0 = 2 \frac{kT}{e} \ln \frac{\sigma}{\sqrt{ekT/2\pi n}}$$

where $n$ is the electrolyte concentration and $e$ the dielectric constant. We recall that the surface charge density $\sigma$ is related to the specific charge by $\sigma = \sigma'/f$, where $f$ denotes the area of one lipid molecule. For a PA bilayer with $f \approx 60 \AA^2$ and $f = 1$, the surface potential $\psi_0$ decreases from 160 mV at $n = 0.1$ M to 100 mV at $n = 1$ M. By this mechanism, an increase in electrolyte concentration, $n$, induces a further increase in the surface charge density, which is strongly dependent on the sodium concentration.

Thus, on the basis of Eq. 3, we have to look for a mechanism that increases the surface charge with increasing ionic strength. A partial answer to this problem comes from the fact that monovalent cations have their greatest effect at about pH 8.5 – 9 (Fig. 2b), i.e., very close to the pK of the reaction

$$\text{HPA}^- \leftrightarrow \text{PA}^+ + \text{H}^+.$$  \[4\]

This process would, in fact, enhance the specific surface charge given by

$$\sigma = \frac{[\text{HPA}^-] + 2[\text{PA}^+]}{[\text{PA}]_T} \times e$$

where $[\text{PA}]_T$ denotes the total lipid concentration. Since this reaction takes place at a negatively charged surface (surface potential $\psi_0$), the association constant, $K$, must be written as $K = K_0 \exp (-\psi_0/kT)$, where $K_0$ is the "intrinsic" association constant and the exponential factor accounts for the electrostatic forces attracting the protons to the negatively charged surface. Hence the degree of dissociation, $\alpha_2$, is given by

$$\alpha_2 = \frac{([\text{HPA}^-] + 2[\text{PA}^+])}{[\text{PA}]_T}.$$  \[5\]

From the Gouy-Chapman theory, an increase in ionic strength lowers the surface potential $\psi_0$. For a highly-charged surface and a 1:1 electrolyte, the relevant equation is

$$\psi_0 = 2 \frac{kT}{e} \ln \frac{\sigma}{\sqrt{ekT/2\pi n}}$$

where $n$ is the electrolyte concentration and $e$ the dielectric constant. We recall that the surface charge density $\sigma$ is related to the specific charge by $\sigma = \sigma'/f$, where $f$ denotes the area of one lipid molecule. For a PA bilayer with $f \approx 60 \AA^2$ and $f = 1$, the surface potential $\psi_0$ decreases from 160 mV at $n = 0.1$ M to 100 mV at $n = 1$ M. By this mechanism, an increase in electrolyte concentration, $n$, induces a further increase in the surface charge density, which is strongly dependent on the sodium concentration.
dissociation of HPA⁻. The resulting increase in $\sigma'$ (Eq. 5) is expected to lower the transition temperature according to Eq. 3. In addition, this reaction will be very dependent on pH.

The proposed dependence can be treated on the basis of Eqs. 4 to 7. In the analysis of this problem we have taken $\gamma$ (see Eq. 3) as an adjustable parameter. The measured curves can be reproduced with $\gamma = 18^\circ\text{C}$ (Fig. 5). This value is in good agreement with the values of $\gamma$ estimated from Eq. 3 and determined from the pH dependence of $T_a$.

Release of $\text{Me}^{++}$. The adsorption of divalent cations to charged bilayers may be governed to a large extent by electrostatic forces (19). In analogy to our discussion of reaction 4, the binding constant for divalent cations is given by $K_{\text{Me}^{++}} = K_{\text{Me}^0} \exp (2e\phi/kT)$, where $K_{\text{Me}^0}$ is the intrinsic binding constant. Invoking Eq. 7 one sees that an increase in ionic strength lowers the value of $K_{\text{Me}^{++}}$ and thereby may trigger the release of divalent cations.

This expectation was tested by the following experiment. $\text{Mg}^{++}$ was added to a salt-free dispersion of PA (2.5 $\times$ 10⁻⁴ M lipid) of pH 9.0 to yield a molar ratio $[\text{Mg}^{++}]/[\text{PA}] = 0.3$; this increased the value of $T_a$ from 47°C to 53°C as expected from our previous experiments (Fig. 4). The subsequent addition of 0.25 M (0.5 M) NaCl lowered the transition temperature to 37°C (36°C). These values are only 3°C (2°C) higher than when these experiments are performed in the absence of $\text{Mg}^{++}$ (Fig. 5). This indicates that a larger part of the initially adsorbed $\text{Mg}^{++}$ has been released by the addition of sodium chloride.

Summary and conclusions

Small changes in the ionic environment (pH, monovalent and divalent cations) may induce gross alterations in the structure of lipid bilayers (ordered ↔ fluid phase transition). As shown for PA, divalent cations stabilize the ordered state (increase in $T_a$) whereas, monovalent cations tend to stabilize the fluid state (decrease in $T_a$). Qualitatively the same behavior is observed for PS above pH 7.

A physicochemical analysis shows that the transition temperature, $T_a$, of charged lipid bilayers decreases with increasing electrical double layer energy $\phi$. For high surface potentials the value of $\phi$ is proportional to the surface charge density, $\sigma$, and independent of the electrolyte concentration. This provides a method to distinguish between charge-neutralization by counterion-adsorption and screening effects within the electrical double layer (change in surface potential $\phi$). The results for the divalent cations $\text{Mg}^{++}$, $\text{Ca}^{++}$, and $\text{Be}^{++}$ can only be interpreted by invoking charge neutralization as the dominant process. For monovalent cations, the ionic strength effect dominates: increasing the electrolyte concentration reduces the surface potential and thereby induces further ionization of surface groups ($\sigma$ increases and hence $T_a$ decreases). The observed release of divalent cations by mono-

The values of $K_{\text{Me}^{++}}$ and $K_{\text{Me}^0}$ for the binding of $\text{Mg}^{++}$ to PA bilayers may be estimated from the initial slope ($dT_i/dm_{\text{Me}^{++}}$ of the curves in Fig. 4. In this estimate we put $\gamma = 23^\circ\text{C}$ and assume that at high pH the binding reaction is $\text{Mg}^{++} + \text{PA}^{--} \rightleftharpoons \text{MgPA}$. The obtained values for $K_{\text{Me}^{++}}$ increase between pH 8 and 10 from 1.6 $\times$ 10⁻⁴ to 7 $\times$ 10⁻⁴ M⁻¹. Correction for the electrostatic term yields $K_{\text{Me}^0} = 0.35$ M⁻¹ independent of the pH. A similar value of $K_{\text{Me}^0}$ (0.1 M⁻¹) has been reported (19) for the association of $\text{Ca}^{++}$ and $\text{Mg}^{++}$ to PS bilayers.

Valent cations is a simple consequence of this reduction in surface potential.

Ionic interactions are critically important for the structure and function of many biomembranes (13). For example, in the visual system divalent cations (released by the primary process) are involved in the signal transmission (20). In view of our results it is conceivable that these cations directly affect the structure of (the negatively charged¹) presynaptic or axonal membranes to enhance or reduce nerve activity.

So far no direct evidence supports the idea that lipid phase transitions are actively involved in biological processes. However, several investigations clearly demonstrate the importance of the lipid structure (fluidity) for several membrane functions (22-24). A structural-functional relationship of this type has been established (25, 26) for intact membranes of Escherichia coli showing thermal phase transitions of the membrane lipids. An abrupt increase in transport rate, eg., $\beta$-galactoside transport, has been observed at the ordered↔fluid transition.

There are striking analogies between our studies and recent ideas about the mechanism of nerve excitation. Tobias (27), Tassaki (28), and Adam (29) assume (a) that abrupt (cooperative) conformational changes occur in the axonal membrane during excitation and (b) that these changes can be triggered by a variation in the molar ratio $\text{Me}^{+}$/Me²⁺. The latter assumption is based on experiments with perfused axons (28), indicating that divalent cations on the outer surface stabilize the resting state whereas monovalent cations tend to induce excitation. In addition, perfused axons exhibit abrupt changes in their electrical state at critical values of the molar ratio $\text{Me}^{+}$/Me²⁺, and of temperature (30, 32).

In view of the high unsaturation of many naturally occurring lipids it is unlikely that entire membranes may show lipid phase transitions. Rather, it is conceivable that certain membrane proteins are surrounded by specific lipids the structure of which is responsive to local changes in pH or the presence of cations. These structural changes in the lipid environment might in turn affect the function of the membrane protein in analogy to the observations with E. coli mutants (25, 26, 31).

We are grateful to Prof. M. Eigen for his support and encouragement.

We thank Miss Sigrid Schröder for her excellent technical assistance.


¹ The main anionic lipid present in nerve membranes is PS with a weight percentage of 15% (21).