# $La^{3+}$-INDUCED FUSION OF PHOSPHATIDYLSERINE LIPOSOMES

## Close Approach, Intermembrane Intermediates, and the Electrostatic Surface Potential

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ABSTRACT The fusion of large unilamellar phosphatidylserine liposomes (PS LUV) induced by $La^{3+}$ has been monitored using the 1-aminonapthalene-3,6,8-trisulfonic acid / *p*-xylenebis(pyridinium bromide) (ANTS/DPX) fluorescence assay for the mixing of aqueous contents. The fusion event is extensive and nonleaky, with up to 95% mixing of contents in the fused liposomes. However, addition of excess EDTA leads to disruption of the fusion products in a way that implies the existence of metastable intermembrane contact sites. The maximal fusion activity occurs between 10 and 100 $\mu$M $La^{3+}$ and fusion can be terminated rapidly, without loss of contents, by the addition of excess $La^{3+}$, e.g., 1 mM $La^{3+}$ at pH 7.4. This observation is explained by the very large intrinsic binding constant ($\sim 10^5$ $M^{-1}$) of $La^{3+}$ to the PS headgroup, as measured by microelectrophoresis. Addition of 1 mM $La^{3+}$ causes charge reversal of the membrane and a large positive surface potential. $La^{3+}$ binding to PS causes the release of a proton. These data can be explained if $La^{3+}$ can chelate to PS at two sites, with one of the sites being the primary amino group. This binding model successfully predicts that at pH 4.5 fusion occurs up to 2 mM $La^{3+}$, due to reduced $La^{3+}$ binding at low pH. We conclude that the general mechanism of membrane fusion includes three kinetic steps. In addition to (*a*) aggregation, there is (*b*) the close approach of the surfaces, or thinning of the hydration layer, and (*c*) the formation of intermembrane intermediates which determine the extent to which membrane destabilization leads to fusion (mixing of aqueous contents), as opposed to lysis. The lifetime of these intermembrane intermediates appears to depend upon $La^{3+}$ binding to both PS sites.

### INTRODUCTION

$La^{3+}$ has been widely used as a probe of $Ca^{2+}$-activated/mediated biological processes because of its ability to replace $Ca^{2+}$ at binding sites (Lettvin et al., 1964; Takata et al., 1966; van Breemen and de Weer, 1970; Heuser and Miledi, 1971; Weiss, 1974; Smith, 1976; Evans, 1983; Mead and Clusin, 1985; Curtis et al., 1986; Segal, 1986). The physicochemical basis of this observation was proposed by Lettvin et al. (1964) based upon the similar ionic radii of these cations. The extra ionic charge on $La^{3+}$ would enhance its attraction to anionic binding sites.

On a more general level, the interactions between cations and specific phospholipids and glycolipids have been studied to construct models of the electrostatic surface structure of membranes (McLaughlin, 1977; Winiski et al., 1986). These studies have shown that the classical Poisson–Boltzmann theory adequately predicts the electrostatic properties provided that the membrane surface charges are properly taken into account. An essential parameter in this theory is the binding constant of each cation to each site on the surface. For phospholipids with a single anionic group, e.g., phosphatidic acid (PA), phosphatidylglycerol (PG), or phosphatidylinositol (PI) at physiological pH, the molecular assignment of the cation binding site is straightforward. For phosphatidylserine (PS), with the triple-charged headgroup at physiological pH, the assignment of cation binding sites is more problematic.

The study of phospholipid vesicle fusion induced by cations requires knowledge of the nature of the binding complex (Düzgüneş and Papahadjopoulos, 1983; Düzgüneş et al., 1987*a*; Bentz and Ellens, 1988). The initial fusion reaction is

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2 \qquad (1)$$

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where $V_1$ denotes the liposome, $V_2$ denotes the aggregated dimer, and $F_2$ denotes the fused doublet (cf. Fig. 9). It has been shown that the dimerization rate constant $C_{11}$ increases with decreased surface charge density, as is expected from a diminished electrostatic repulsion between the liposomes (Nir et al., 1980, 1983; Bentz et al., 1983*b*, 1985*a*; Wilschut et al., 1985*a* and *b*; Meers et al., 1986). On the other hand, the fusion rate constant $f_{11}$ is quite sensitive to the identity, as well as the amount, of the bound cation (Wilschut et al., 1981; Düzgüneş et al., 1981; Bentz et al., 1983*b*, 1985*a*; Bentz and Düzgüneş, 1985).

These studies have elucidated how the cation-phospholipid headgroup complex promotes the destabilization of the apposed membranes and how that destabilization is propagated. To establish some intermembrane contact, a thinning of the aqueous layer between the apposed membranes must be accomplished, i.e., the repulsive hydration force must be surmounted (Rand, 1981; Rand and Parsegian, 1984, 1986; Bentz et al., 1985*a*; Wilschut et al., 1985*a* and *b*; Evans and Needham, 1986). Once achieved, the molecular contact between the apposed outer monolayers may or may not result in fusion.

Biologically relevant fusion implies a quantitative mixing of contents, i.e., little or no leakage. The $Ca^{2+}$-induced fusion of PS liposomes has been the prototypical system for developing fluorometric assays for mixing of bilayer components and aqueous contents during fusion. Ironically, the same system has provoked considerable disagreement as to the interpretation of this fusion event and as to the relevance of this system for developing a biophysical theory of membrane fusion (for reviews see Rand and Parsegian, 1984, 1986; Bentz and Ellens, 1988; Düzgüneş and Bentz, 1988). The essence of the problem is that very large PS multilamellar liposomes (~10 μm diameter) in low ionic strength media (2 mM $Na^+$) show evidence of both fusion- and contact-mediated rupture or lysis (Rand et al., 1985; Kachar et al., 1986), whereas PS LUV (~0.1 μm diameter) in intermediate ionic strengh media (20–200 mM $Na^+$) show complete mixing of contents followed later by collapse and leakage (Wilschut et al., 1980, 1983, 1985*b*; Bentz et al., 1983*a*, 1985*a*; Bentz and Düzgüneş, 1985).

The fact that the $Ca^{2+}$-induced fusion does cause eventual collapse has led to one model in which adhesion of the liposomes promotes tension on the membranes (while overcoming the hydration force) which leads to their rupture (Rand and Parsegian, 1984, 1986; Rand et al., 1985; Kachar et al., 1986; Evans and Needham, 1986). When that rupture occurs in the area of contact, there is fusion; otherwise, there is lysis. This model does not ascribe any role to specific intermembrane intermediates between the apposed membranes.

In this report, we extend the basis of discussion of the PS liposome fusion mechanism by observing the effects of $La^{3+}$, which differ from those of $Ca^{2+}$ in three essential respects. (*a*) There is no collapse (leakage) even after extensive fusion. To be consistent with the model just described, rupture due to enhanced membrane tension must occur only in the area of interliposomal contact, which certainly suggests the existence of intermembrane intermediates. (*b*) At pH 7.4, fusion is disrupted by addition of excess EDTA in a fashion which implies that these intermembrane intermediates are long-lived. (*c*) Fusion is initiated by very low $La^{3+}$ concentrations, ≥2 μM, and is abolished by addition of excess $La^{3+}$, e.g., 1 mM at pH 7.4. This last observation is explained by a very large $La^{3+}$-PS binding constant. Excess $La^{3+}$ binding reverses the electrostatic surface potential and abolishes close apposition. Using microelectrophoresis, we have measured the $La^{3+}$-PS binding constant (~$10^5$ $M^{-1}$) and have shown that it is extremely sensitive to pH. Based upon this result, the simplest molecular mechanism of $La^{3+}$ binding to the PS headgroup must involve two sites, one of which is the primary amino group. Chelation to this site is inhibited at low pH. The intermembrane intermediates are much longer lived (more stable) when $La^{3+}$ is bound to both sites.

## MATERIALS AND METHODS

Bovine brain phosphatidylserine (PS) was purchased from Avanti Polar Lipids (Birmingham, AL), stored at −70°C, and used within the three-month shelf-life quoted by the supplier. Ultrapure calcium chloride ($CaCl_2 \cdot 4H_2O$) was obtained from E. Merck (Darmstadt, FRG), $LaCl_3 \cdot 6H_2O$ from Alpha Products (Danvers, MA) and $TbCl_3 \cdot 6H_2O$ from AESAR Rare Earth Products (Seabrook, NH). Due to the hygroscopic nature of these compounds, the stock concentrations for these solutions were determined using a vapor pressure osmometer (Wescor, Inc., Logan, UT) on the assumption that the ions completely dissociate in solution. Calcium stocks were titrated to pH 7.4 in the presence of 10 mM *N*-[Tris (hydroxymethyl) methyl]-2-aminoethane sulfonic acid (TES). Lanthanum stocks were untitrated and used at pH 4.8 due to the insolubility of the hydroxides. It is worth recalling that both $La^{3+}$ and other multivalent cations in solution are subject to hydrolysis (Prados et al., 1974; Cotton and Wilkinson, 1980). Thus, the stock solutions must be kept at low pH to avoid uncertainties in the true cation concentrations. The fluorophore 1-aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and its collisional quencher *N,N'-p*-xylene*bis*(pyridinium bromide) (DPX) were obtained from Molecular Probes (Eugene, OR). Dipicolinic acid (DPA) was obtained from Sigma Chemical Co. (St. Louis, MO), and nitrilotriacetic acid (NTA) from Aldrich Chemical Co. (Milwaukee, WI). 3(*N*-Morpholino)propane sulfonic acid (MOPS) was purchased from Research Organics, Inc. (Cleveland, OH). Octaethyleneglycoldodecyl ether, $C_{12}E_8$ (Calbiochem-Behring Corp., La Jolla, CA), was recrystallized from hexane and filtered (0.2 μm).

Reverse-phase evaporation vesicles (REV) were prepared from 10 μmol PS as described by Szoka and Papahadjopoulos (1978). The ANTS/DPX fusion liposomes contained either (*a*) 25 mM ANTS, 40 mM NaCl, 10 mM MOPS; (*b*) 90 mM DPX, 10 mM MOPS; or (*c*) 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, 10 mM MOPS. A buffer containing 115 mM NaCl, 10 mM MOPS was used for column chromatography and fluorescence experiments. Liposomes prepared for Tb/DPA fusion and leakage experiments contained (*a*) 2.5 mM $TbCl_3$, 50 mM NTA, 50 mM MOPS; (*b*) 50 mM DPA, 20 mM NaCl, 50 mM MOPS; or (*c*) 1.25 mM $TbCl_3$, 25 mM NTA, 25 mM DPA, 10 mM NaCl, 50 mM MOPS. For the comparison of the ANTS/DPX and Tb/DPA assays, the ANTS/DPX liposomes were prepared as described above except that 50 mM MOPS was used. A corresponding chromatography and experimental buffer with 100 mM NaCl, 50 mM MOPS, pH 7.4 was used for relevant experiments. All solutions were titrated to pH 7.4 and made iso-osmotic with NaCl.

The liposomes were extruded three times through a 0.1-μm polycarbonate membrane (Nuclepore Corp., Pleasanton, CA) under 80 psi argon. The liposomes were separated from unencapsulated material on Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). Phospholipid concentrations were determined by phosphate analysis (Bartlett, 1959). The liposomes were characterized by quasielastic light scattering (model N4, Coulter Electronics Inc., Hialeah, FL) and by trapped-volume determinations (Ellens et al., 1985). ANTS-containing liposomes had diameters of 150 nm and trapped volumes of 5.0 μl/μmol phospholipid, which is consistent with large unilamellar vesicles.

Fluorescence and light scattering measurements were recorded simultaneously on a SPEX Fluorolog 2 (SPEX Industries, Edison, NJ) equipped with two 90° photon-counting emission channels set at 0.5 s integration times. The ANTS/DPX contents-mixing and leakage experiments were done as described by Ellens et al. (1985). Excitation was at 360 nm and fluorescence emission was measured using a Schott GG 435 cutoff filter (Melles Griot, Irvine, CA). The Tb/DPA assay was performed as described by Wilschut et al. (1980). Excitation was at 276 nm and fluorescence emission was measured using a 3-69 filter (Corning Glass Works, Corning, NY).

For the ANTS/DPX assay, the fluorescence scale was calibrated for a 1/1 mixture of ANTS and DPX liposomes at 10 μM total phospholipid, unless otherwise stated. Data were plotted as percent fluorescence versus time. The 0% fluorescence level was determined from 10 μM liposomes containing coencapsulated ANTS/DPX. This level corresponds to 100% fusion and 0% leakage. The 100% fluorescence level was determined using 5 μM ANTS liposomes with 5 μM DPX liposomes before fusion. This level corresponds to 0% fusion and 100% leakage. The 100% level for the leakage experiments was confirmed by detergent lysis of the ANTS/DPX liposomes. The disodium salt of EDTA (Fisher Scientific Co., Pittsburgh, PA) used in certain experiments was added from a 400 mM stock titrated to pH 7.4 with 400 mM TES. For fusion and leakage experiments at pH 4.5, a 115 mM NaCl solution titrated with HCl was used to avoid the presence of acetate and citrate buffers. The pH was checked after the addition of liposomes and following the addition of the various cationic and chelating solutions.

Electrophoretic measurements were done with a Mark II apparatus (Rank Brothers, Bottisham, UK), employing a four-probe flat cell immersed in a thermostatted bath whose temperature was controlled to 25.00 ± 0.05°C with a proportional temperature controller. The cell was oriented so that any precipitated vesicles fell on a hydrodynamically unimportant surface. The electric field was applied via the current electrodes by a current source at 0.5 mA, and voltage was monitored across the voltage electrodes by a Keithley 614 digital electrometer. Each electrophoresis run consisted of 20 mobility readings, five in each direction at each stationary layer. The data were discarded if the average mobilities at the two stationary layers differed by >15%. Runs for each set of conditions were reproduced at least twice and weighted averages of the mobilities taken. Zeta potentials were calculated from the mobilities by use of the Helmholtz–Smoluchowski equation. Multilamellar vesicles, ~1–10 μM diameter, were formed by hydrating PS in 0.1 M NaCl/10 mM MOPS at 4°C overnight and vortexing before use.

The electrophoretic experiments at pH 7.4 (10 mM MOPS) were performed with platinum electrodes. To study whether $La^{3+}$ binding to MOPS was significant, certain experiments were run "without buffer," <10 μM MOPS. Without buffer, it was found that the Pt electrodes generated protons during a run (which took ~10 min) and lowered the pH significantly. With palladium electrodes, there was little or no shift in pH during a run, ≤±0.2 pH units. Thus, for the low pH runs, Pd electrodes were used. Control experiments with buffer at pH 7.4 showed that the Pd and Pt electrodes gave identical results.

## RESULTS

Fusion is monitored via the mixing of encapsulated contents using the ANTS/DPX assay. Briefly, the extent of quenching of ANTS fluorescence is equal to the fraction of ANTS and DPX which have mixed within the fused liposomes. Because we begin with equal concentrations of ANTS-containing and DPX-containing liposomes, the first round of fusion to doublets leads to 50% quenching because only half of the fused doublets contain both ANTS and DPX. Leakage during or after fusion would diminish the extent of fluorescence quenching, because ANTS is not quenched by DPX that has leaked into the medium (Ellens et al., 1984, 1985).

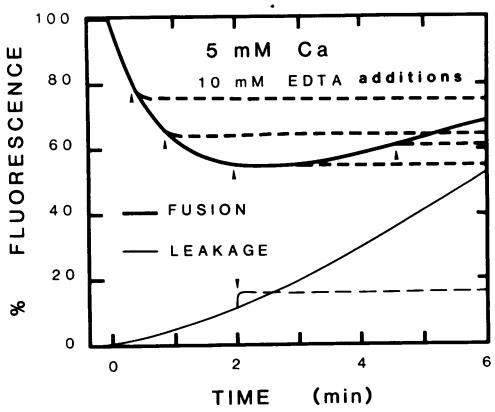


FIGURE 1. Fusion and leakage of 10 μM PS LUV in 100 mM NaCl/10 mM MOPS at pH 7.4 induced by 5 mM $Ca^{2+}$ as monitored by the ANTS/DPX fluorescence assay for leakage and for the mixing of aqueous contents. Leakage of contents is shown by the lower solid line. The ANTS fluorescence is quenched inside the liposomes by DPX. If all of the liposomes fused once to doublets, with no leakage, then the fusion fluorescence would be 50% because only half of the fused doublets would contain both ANTS and DPX. The effect of adding excess (10 mM) EDTA to the medium (using identical sample preparation) at various times is shown by dashed lines. The maximum quenching (or minimum fluorescence) demarks the time (~2 min) at which leakage of contents exceeds the mixing of contents.

Fig. 1 shows the $Ca^{2+}$-induced fusion of PS LUV as monitored by the ANTS/DPX assay. Maximal quenching is ~40%, which occurs at 2 min. Thereafter, leakage and collapse of the fusing liposomes lead to a decrease in the amount of mixed contents and in the observed fluorescence quenching (Wilschut et al., 1980, 1983; Bentz et al., 1983*a*, 1985*a*). Also shown in the figure is the effect of adding excess EDTA to the system at various times. Removal of $Ca^{2+}$ causes an immediate cessation of both fusion and leakage.

### Nonleaky Fusion Induced by $La^{3+}$

Fig. 2 *A* shows the fusion of 10 μM of PS LUV induced by various $La^{3+}$ concentrations, as noted in μM units. The $La^{3+}$-induced fusion differs from that induced by $Ca^{2+}$ in at least three ways. (*a*) Fusion occurs at micromolar $La^{3+}$ concentrations rather than the millimolar concentrations of $Ca^{2+}$ normally required. (*b*) Fusion is maximal at 10–100 μM $La^{3+}$ and is inhibited at higher $La^{3+}$ concentrations. (*c*) Fusion is extensive and nonleaky. The extent of contents mixing (≥90% quenching) requires a minimum of four liposomes per fusion product without leakage

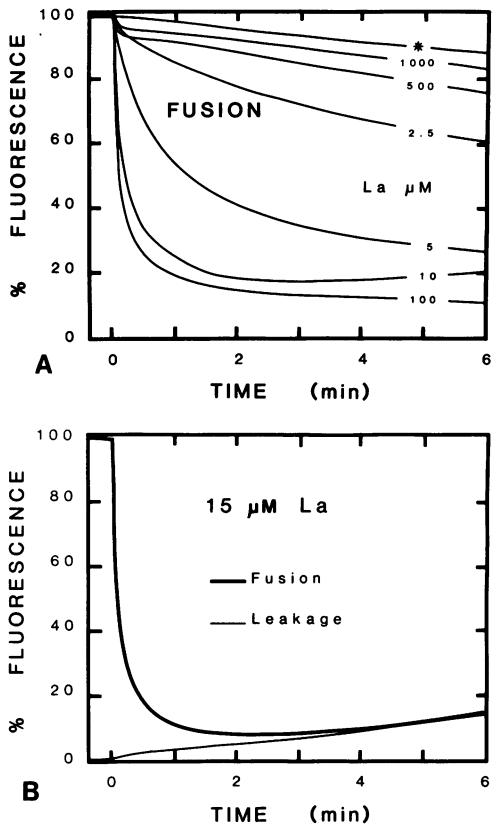


FIGURE 2 (*A*) Fusion of 10 μM PS LUV in $Na^+$ buffer induced by various $La^{3+}$ concentrations, noted in micromolar units beside each curve. The initial rates of mixing of contents increase as the $La^{3+}$ concentration increases from 2.5 to 10 μM. From 10 to 100 μM $La^{3+}$, the fusion curves are quite similar, indicating that maximal rates of aggregation and fusion have been achieved. Above 200 μM $La^{3+}$, fusion is inhibited. As discussed in the text, this reflects the inability of the liposomes to aggregate because $La^{3+}$ binding to the liposomes produces large positive surface potentials. The slow decline in fluorescence in the presence of 1 mM $La^{3+}$ is not due to fusion of the liposomes, as the curve denoted (*) shows the effect of 1 mM $La^{3+}$ on 10 μM of pure ANTS liposomes. Aside from the small injection offset, the curves are identical. (*B*) Fusion (*heavy line*) and leakage (*light line*) for 10 μM PS LUV induced by 15 μM $La^{3+}$. Leakage is monitored by coencapsulating ANTS and DPX. Leakage results in dilution of DPX and relief of its collisional quenching of ANTS. The subsequent recovery of fluorescence from the fusion products is due to slow leakage.

(Düzgüneş and Bentz, 1988). On the other hand, light scattering and turbidity measurements did not indicate very large aggregates (data not shown).

Whereas the ANTS/DPX assay has been well characterized (Ellens et al., 1984, 1985, 1986*a* and *b*; Bentz et al., 1985*b*, 1987; Düzgüneş et al., 1985, 1987*b*; Düzgüneş and Bentz, 1988), such an extensive degree of contents mixing is rare for pure PS LUV (however, see Fig. 2 of Bentz et al., 1985*a*). To verify that the fluorescence signal was due solely to fusion, we have also used the Tb/DPA fusion assay (Wilschut et al., 1980). With PS LUV both assays gave essentially the same results for both the kinetics and the large extent of contents mixing. The only difference is that the ANTS/DPX assay registered a slightly faster fusion rate than did the Tb/DPA assay. This effect has also been noted with $Ca^{2+}$-induced fusion of PS LUV and has been discussed (Ellens et al., 1985; Düzgüneş and Bentz, 1988; Düzgüneş et al., 1987*b*).

To determine the leakage of contents induced by $La^{3+}$, we monitored the relief of fluorescence quenching of coencapsulated ANTS/DPX (Ellens et al., 1984). Fig. 2 *B* shows the fusion and the leakage signals induced by 15 μM $La^{3+}$. The subsequent increase in the fusion fluorescence signal is matched by the leakage of contents. The addition of detergent ($C_{12}E_8$) led to disruption of the liposomes and complete relief of ANTS quenching by DPX.

The lack of fusion at higher $La^{3+}$ concentrations is novel, as the fusion rate had always been found to increase with the amount of bound divalent cation (Bentz & Düzgüneş, 1985). Fig. 3 shows the effect of initiating fusion with 15 μM $La^{3+}$ and then adding an additional 1 mM $La^{3+}$ at subsequent times. In all cases, addition of "excess" $La^{3+}$ caused an immediate cessation of fusion. Leakage, to the small extent observed, was also immediately stopped by the addition of excess $La^{3+}$ (data not shown). Unlike the data in Fig. 1, where the $Ca^{2+}$-induced fusion was stopped by removing the bound $Ca^{2+}$, here $La^{3+}$ is still bound to the PS, but the membranes are no longer closely apposed. A simple explanation of these data is that the binding of $La^{3+}$ to PS leads to a complex having net positive charge. When enough $La^{3+}$ is bound, the positive electrostatic surface potential becomes large enough to force the membranes apart. Flocculates are still observed, indicating that complete dispersal of aggregates does not occur. Dynamic light scattering indicates <10 liposomes per fusion product (data not shown).

## Binding of $La^{3+}$ to PS Membranes

To examine the above hypothesis, we measured the electrophoretic mobility of large (≥10 μm) multilamellar PS liposomes (MLV) in the presence of $La^{3+}$. Using the Helmholtz–Smoluchowski relation one can convert the

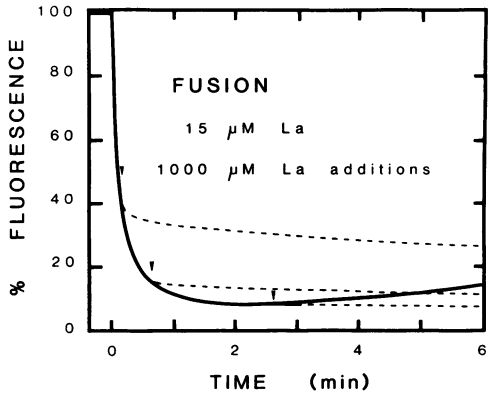


FIGURE 3 Fusion of 10 μM PS LUV induced by 15 μM $La^{3+}$ (*solid line*) ceases abruptly (*dashed lines*) upon addition of 1 mM $La^{3+}$ at various times, shown by arrows (▼).

electrophoretic mobility to a zeta potential, i.e., the electrostatic potential at the shear surface of the particle (Eisenberg et al., 1979; McLaughlin et al., 1981). Fig. 4 *A* shows these zeta potentials under conditions similar to those of the fusion experiments (■, pH 7.4 with 0.1 M NaCl/10 mM MOPS). It is quite obvious that at low $La^{3+}$ concentrations (≤10 μM), the zeta potential is large and negative (<−30 mV). Likewise, above 100 μM $La^{3+}$ the zeta potential is large and positive (≥20 mV). Therefore, the range of $La^{3+}$ concentrations where fusion is observed corresponds exactly to the range where the magnitude of the zeta potential is small.

The solid lines in Fig. 4 *A* are theoretically calculated zeta potentials, assuming that both $Na^+$ and $La^{3+}$ bind specifically to the PS headgroup (denoted $P^-$) following the mass action laws:

$$Na^+ + P^- \overset{K_{11}}{\rightleftharpoons} PNa$$

$$La^{3+} + P^- \overset{K_{31}}{\rightleftharpoons} PLa^{2+} \qquad (2)$$

In this model $La^{3+}$ and $Na^+$ compete for the same site and there is a 1:1 binding stoichiometry between the cations and the PS headgroup. Later, we will refine this model. The equations needed to solve the Poisson–Boltzmann equation for the electrostatic surface potential, including cation binding, can be found in the Appendix. The parameters used in the calculation are given in the figure legend. The shear surface is assumed to lie 2 Å from the membrane surface (Eisenberg et al., 1979; McLaughlin et al., 1981). Assuming another distance for the shear surface would only alter the values of the fitted $La^{3+}$ binding constant (McLaughlin et al., 1983).

At pH 7.4 (■, Fig. 4 *A*) with high $La^{3+}$ concentrations (≥100 μM), a $La^{3+}$-PS binding constant $K_{31} = 10^5\ M^{-1}$ fits the electrophoretic data quite well. At low $La^{3+}$ concentrations (≤10 μM), the zeta potential is much too negative to be fitted by this binding constant, or even a constant two orders of magnitude smaller. The problem at low $La^{3+}$ concentrations is that the free $La^{3+}$ concentration is probably much less than that inferred from the total amount added. With the calculated curves, it is assumed that the free $La^{3+}$ concentration results from the total amount added, i.e., that the amounts of $La^{3+}$ bound to membrane sites, as well as to the glass walls, buffer, and electrodes, do not deplete the aqueous concentration. Although the total PS concentration is 10 μM, the amount of lipid present on the external monolayer of the MLV is not known, nor is the amount of $La^{3+}$ lost to interactions with other surfaces. These uncertainties preclude any rigorous calculation of the free $La^{3+}$ concentrations (Nir, 1984). Any such corrections would increase the agreement between the data and the curve calculated for the binding constant $K_{31} = 10^5\ M^{-1}$. In the discussion of Table II (see below), we note that

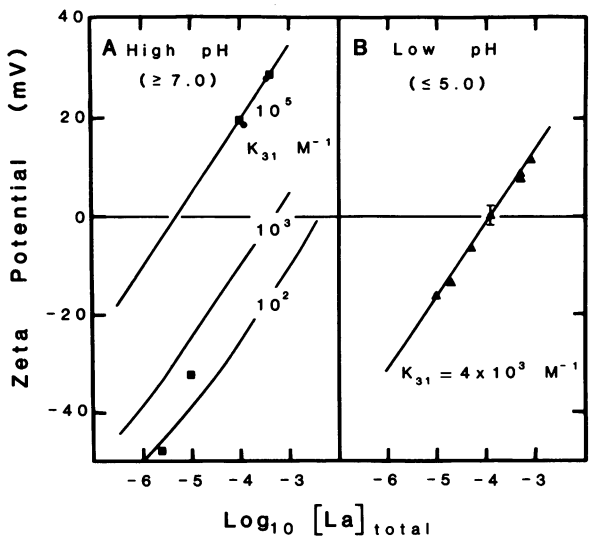


FIGURE 4 Zeta potentials for 10 μM large PS MLV as measured by microelectrophoresis in the presence of 0.1 M NaCl and various total $La^{3+}$ concentrations. The standard deviations are smaller than the symbols, except as shown. Zeta potentials were calculated from electrophoretic mobilities by the Helmholtz–Smoluchowski equation. (*A*) Effect at neutral pH with 10 mM MOPS (■) where there is surface-charge reversal between 10 and 100 μM $La^{3+}$. Also shown are data points for cases where there is no MOPS (●, ≤10 μM MOPS), made by diluting the MLV stock into 100 mM NaCl initially titrated with NaOH to pH 9.5. At the end of the run the pH was ≥8.0, due to the generation of protons by the Pt electrodes. Solid lines are calculated from the Gouy-Chapman-Stern theory using a $Na^+$ binding constant, $K_{11} = 1\ M^{-1}$, and a $La^{3+}$ binding constant, $K_{31} = 10^5$, $10^3$, or $10^2\ M^{-1}$, as noted in the figure, as well as the assumption that all cations bind competitively (with a 1:1 stoichiometry) to the same site. The shear surface was assumed to be 2Å from the membrane surface. (*B*) shows the zeta potentials at low pH (≤5.0 during the run). The theoretical curve is calculated assuming that the $La^{3+}$ binding constant is reduced to $K_{31} = 4 \times 10^3\ M^{-1}$. The more detailed model described in Fig. 8 and evaluated in Table II would give essentially identical predictions for the zeta potentials.

5 μM exposed PS would reduce 2 μM total $La^{3+}$ to 0.2 μM free $La^{3+}$.

$La^{3+}$ binding to MOPS was weak, if it occurred at all. The data points (●) in Fig. 4 *A* show the case of MLV without buffer, i.e., ≤10 μM MOPS. Clearly, these data are essentially identical to the case of 10 mM MOPS. Thus, it is very unlikely that the low free $La^{3+}$ concentrations in 10 mM MOPS, at and below 10 μM total $La^{3+}$, is due to binding to MOPS.

Determination of whether the "extraneous" binding sites were due to PS, e.g., leakage of the $La^{3+}$ into the MLV, would require using much less lipid, which was not feasible for the electrophoresis experiments. We do note, however, that for the MLV to be negatively charged, no more than 3.3 μM $La^{3+}$ can be sequestered per 10 μM PS. With 10 μM total $La^{3+}$, this leaves at least 6.6 μM free $La^{3+}$, which would give a predicted zeta potential of +2 mV, rather than the experimentally measured −32 mV. For these reasons, we suspect that the "extraneous" binding sites belong to the microelectrophoresis cell.

## Release of $H^+$ by $La^{3+}$ Binding to PS

It has been shown that the binding of $La^{3+}$ (Hammoudah et al., 1981) and some divalent transition metals (Puskin, 1977) to PS releases a proton. We have speculated that $Mn^{2+}$ and $Ni^{2+}$ may form a chelation complex with the carboxyl and amino groups of the PS headgroup, making a five atom ring (Bentz and Düzgüneş, 1985). Such complexes would require the release of a proton and are known to occur with similar compounds (Sigel and Martin, 1982). We have found the pH to decrease following $La^{3+}$ addition to PS LUV suspended in 0.1 M NaCl without buffer (data not shown). To address this point, we performed some electrophoresis experiments in the absence of MOPS, using a 100 mM NaCl solution and adjusting the pH to 4.5 before the run with HCl/NaOH. The pH of the solution was also measured after the run.

In Fig. 4 *B*, we show the zeta potentials obtained for the PS MLV when the electrolyte, with no MOPS buffer, was pretitrated to ~pH 4.5. The pH after each run was 4.5 ± 0.2. It is obvious that the zeta potential is quite sensitive to the pH. With 100 $\mu$M $La^{3+}$ it is ~20 mV less positive at pH 4.5 than at pH 7.4. The data fit the theoretical curve for a binding constant of $K_{31}$ = 4,000 $M^{-1}$ very well, even at the lower $La^{3+}$ concentrations. This would indicate that the extraneous binding sites, proposed previously to explain the discrepancy at the low $La^{3+}$ concentrations at pH 7.4, are inhibited from binding $La^{3+}$ at pH 4.5.

An obvious control for this experiment is to show that this pH sensitivity is in fact due to the La–PS binding complex, i.e., that in the absence of $La^{3+}$ the electrophoresis is insensitive to pH in this range. Papahadjopoulos (1968) showed that the zeta potential of PS is essentially constant in the pH range 4–10. In Table I, we show the measured zeta potentials for the MLV in 0.1 M NaCl in the presence and absence of both MOPS and EDTA at several pH values. Our value of −58.3 mV in 10 mM MOPS and 0.1 mM EDTA is essentially the same as that found by Eisenberg et al. (1979) and McLaughlin et al. (1981). Removing the MOPS, with a final pH of 6.6, had no effect on the zeta potential. Removing the EDTA and the MOPS caused the zeta potential to fall slightly, presumably due to the presence of trace multivalent cations in the NaCl buffer (Eisenberg et al., 1979). However, the zeta potential is otherwise indifferent to whether the pH is high (7.5) or low (4.5). Thus, the relative inhibition of $La^{3+}$ binding to PS at low pH, at least at the higher $La^{3+}$ concentrations, is likely due to the inhibition of amine deprotonation required to complete the $La^{3+}$ chelation complex.

TABLE I
EFFECT OF BUFFER ON THE ZETA POTENTIAL OF PHOSPHATIDYLSERINE MLV*

| [MOPS] | [EDTA] | pH‡ | Zeta§ |
|---|---|---|---|
| *mM* | *mM* | | *mV* |
| 10 | 0.1 | 7.4 | −58.3 ± 0.5 |
| 0 | 0.1 | 6.6 | −58.3 ± 0.8 |
| 0 | 0 | 7.5 | −51.8 ± 0.9 |
| 0 | 0 | 4.5 | −51.2 ± 0.5 |

*The medium contains 100 mM NaCl and 10 $\mu$M total lipid at 25°C.
‡pH is measured after the run and differs from the reading taken before the run by ≤0.2.
§Details on the electrophoretic measurements can be found in Materials and Methods.

## Fusion at Low pH with Higher $La^{3+}$ Concentrations

Regardless of the specific atomic structure of the La–PS complex, if aggregation is inhibited when the zeta potential exceeds ~30 mV in magnitude, then from Fig. 4 *B* we would predict that significant fusion should occur at 1 mM $La^{3+}$ when the pH is low and the zeta potential is ~10 mV. In Fig. 5, we show the extensive and nonleaky fusion induced by 15 $\mu$M or 1 mM $La^{3+}$ at pH 4.5. In these experiments, the medium is titrated to pH 4.5 in the absence of buffer, i.e., ≤10 $\mu$M MOPS. Given our presumption of $La^{3+}$ binding to carboxyl groups, we chose not to use any buffering ions here. Fusion is also observed with 2 mM $La^{3+}$ (data is essentially identical to that for 1 mM $La^{3+}$). Therefore, the correlation between binding, the zeta potential, and fusion appears correct.

Adding excess EDTA causes immediate cessation of fusion with no loss of contents, as was observed with the $Ca^{2+}$-induced fusion. It is important to note here that EDTA addition raises the pH to 7.4, because the EDTA solution is buffered to that pH. We will see in Fig. 7 that this behavior is in stark contrast to that found when the pH remains 7.4 throughout the process.

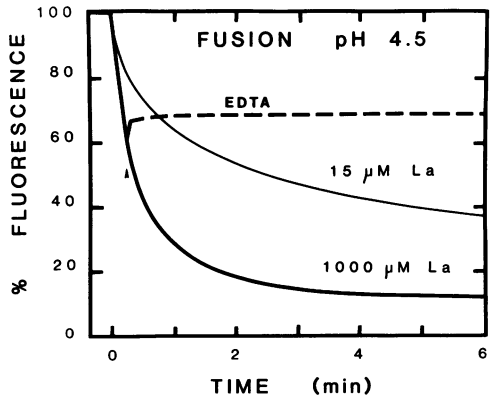


FIGURE 5 Fusion of 10 $\mu$M PS LUV induced by 15 $\mu$M or 1 mM $La^{3+}$ at pH 4.5. The inhibition of $La^{3+}$ binding in the low pH electrolyte allows fusion to occur at 1 mM $La^{3+}$ (compare with Fig. 2). The curve at 2 mM $La^{3+}$ is essentially identical to that for 1 mM $La^{3+}$. This result was predicted from the electrophoresis data and the hypothesis that high concentrations of $La^{3+}$ inhibit fusion by inhibiting aggregation due to large positive surface potentials at pH 7.4. Addition of 10 mM EDTA, final pH 7.4, stops fusion with a small burst of leakage (*dashed line*).

## Interactions Between the Cations During Fusion

We will propose a model for $La^{3+}$ binding to PS derived from studies of the chelation complexes found with serine groups and certain divalent transition metals, e.g., $Mn^{2+}$ but not $Ca^{2+}$, because similar studies have not yet been made for $La^{3+}$. To facilitate the plausibility of this argument, we will show that the fusion induced by $La^{3+}$ is more similar to that induced by $Mn^{2+}$ than to that induced by $Ca^{2+}$.

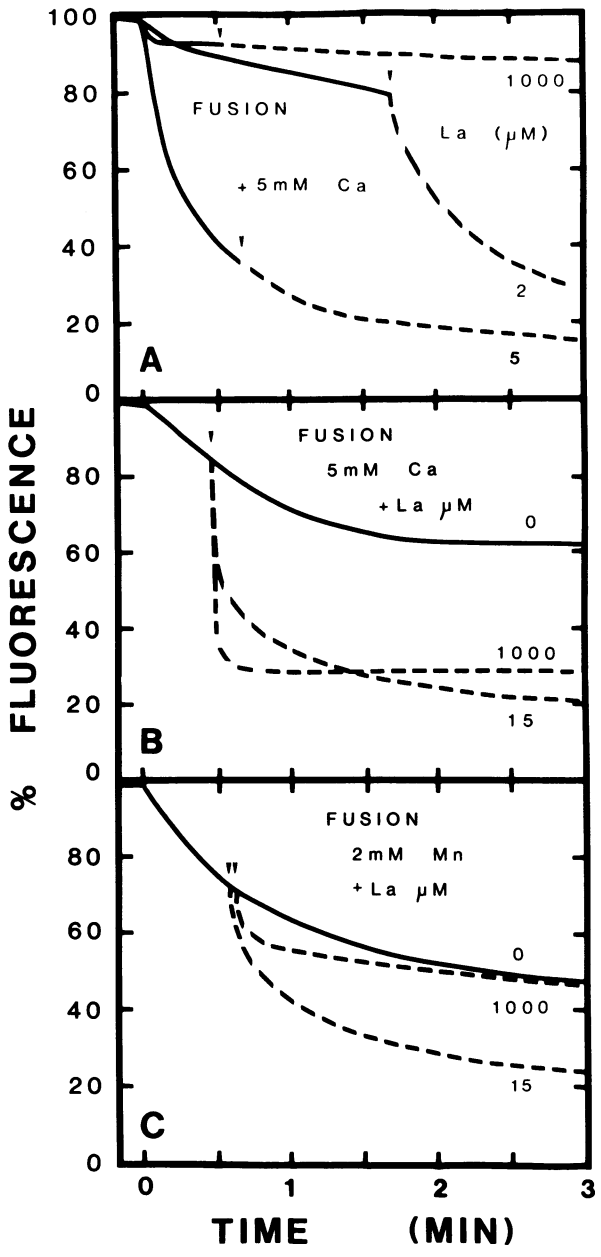


FIGURE 6 Fusion of PS LUV induced by $La^{3+}$, $Ca^{2+}$, and $Mn^{2+}$ at neutral pH. (*A*) Fusion signal for various $La^{3+}$ concentrations (2, 5, and 1,000 μM) and the effect of subsequently adding 5 mM $Ca^{2+}$ (*dashed lines*). (*B*) Fusion signal for 5 mM $Ca^{2+}$ and the effect of adding 15 or 1,000 μM $La^{3+}$. (*C*) Fusion signal from 2 mM $Mn^{2+}$ and the effect of adding 15 or 1,000 μM $La^{3+}$. These data suggest that $La^{3+}$ and $Mn^{2+}$ compete for the same binding sites, whereas $Ca^{2+}$ binds to another site.

Fig. 6 shows the fusion induced by several cations and the effect of subsequent additions of other cations. In Fig. 6 *A*, fusion is initiated by various $La^{3+}$ concentrations (shown in μM units) and 5 mM $Ca^{2+}$ is added subsequently (▼). With 1 mM $La^{3+}$, which shows no fusion, the addition of 5 mM $Ca^{2+}$ produced no fusion and no leakage. In fact, calculations presented in Table II (see below) show that no $Ca^{2+}$ would be expected to bind to the membrane under these conditions. Starting with 5 μM $La^{3+}$, the subsequent addition of 5 mM $Ca^{2+}$ produced no alteration in the fusion curve (see also Fig. 2 *A*). However, with 2 μM $La^{3+}$, where fusion is slow, adding 5 mM $Ca^{2+}$ sharply enhanced fusion. The fusion then proceeded to the maximal extent found for 5 μM $La^{3+}$. Whereas $Ca^{2+}$ promoted the aggregation of the liposomes, the fusion was completely dominated by bound $La^{3+}$, as the calculations in Table II predict.

In Fig. 6 *B*, we show the effect of adding $La^{3+}$ to a system already fusing with 5 mM $Ca^{2+}$. After 20 s of fusion in $Ca^{2+}$, the addition of 15 μM $La^{3+}$ promoted fusion to a maximal level of contents mixing typical of $La^{3+}$. Light scattering showed that aggregation continued. The most interesting result here is that adding excess (1 mM) $La^{3+}$ also causes an immediate increase in contents mixing to a maximal level. In this case, light scattering showed that further aggregation was halted. This is consistent with $La^{3+}$ causing fusion of aggregates, before enough $La^{3+}$ is bound to inhibit close approach electrostatically.

In Fig. 6 *C*, we show the fusion induced by $Mn^{2+}$ and its interaction with subsequently added $La^{3+}$. 2 mM $Mn^{2+}$ produced substantial fusion. Adding 15 μM $La^{3+}$ after 30 s of $Mn^{2+}$-induced fusion led to the extensive mixing of contents found for $La^{3+}$ alone. On the other hand, adding 1 mM $La^{3+}$ after 30 s stopped the fusion with an initial increase in the level of contents mixing. This increase was substantially less than that found when $Ca^{2+}$-induced fusion was interrupted by 1 mM $La^{3+}$.

We also examined the interaction between $Mn^{2+}$ and $Ca^{2+}$, which have comparable binding constants to PS, 12 $M^{-1}$ for $Ca^{2+}$ and 25 $M^{-1}$ for $Mn^{2+}$ (McLaughlin et al., 1981). It was observed that adding 5 mM $Ca^{2+}$ after 30 s of $Mn^{2+}$-induced fusion (with either 2 mM or 5 mM $Mn^{2+}$) did not affect the fluorescence signal appreciably (data not shown).

Overall, the fusion induced by $Mn^{2+}$ and its subsequent behavior upon addition of excess $La^{3+}$ is quite similar to that of $La^{3+}$-induced fusion. The inability of 5 mM $Ca^{2+}$ to alter the fusion induced by 2 mM $Mn^{2+}$ is curious. If both $Ca^{2+}$ and $Mn^{2+}$ bound to the same site, then we would calculate 0.21 Ca bound per PS and 0.18 Mn bound per PS, using 12 $M^{-1}$ as the binding constant for $Ca^{2+}$ and 25 $M^{-1}$ as the binding constant for $Mn^{2+}$ (McLaughlin et al., 1981). This is certainly enough bound $Ca^{2+}$ to induce fusion without $Mn^{2+}$ present (Bentz and Düzgüneş, 1985).

These facts taken together strongly imply that $La^{3+}$ and $Mn^{2+}$ bind to the same site on PS, which is different from the $Ca^{2+}$ binding site.

## Disruption of Intermembrane Intermediates at pH 7.4

Our final result concerns the effect of chelation of $La^{3+}$ by EDTA at pH 7.4. As shown in Figs. 1 and 5, addition of EDTA to liposomes fusing in $Ca^{2+}$ or in $La^{3+}$ at low pH stopped the fusion with no significant destabilization and leakage. Fig. 7, on the other hand, shows that at pH 7.4 the fusion products of $La^{3+}$ are strongly destabilized by EDTA. In fact, adding EDTA late in the process leads to complete release of contents. When EDTA is added to liposomes incubated in 1 mM $La^{3+}$, there is a small release (<10%) of contents. In fact, if the $La^{3+}$-induced fusion is first stopped by addition of excess $La^{3+}$, then the addition of EDTA results in the same small release of contents. This proves that merely chelating the $La^{3+}$ from the PS membrane surface (even after some fusion has occurred) does not induce destabilization. The small release probably occurs during the period when the EDTA chelation reaction corresponds to 10–100 $\mu$M free $La^{3+}$ and some rapid aggregation and fusion is followed by brief destabilization. In Fig. 5, the EDTA chelation of $La^{3+}$ at pH 4.5, producing a final pH of 7.4, causes no leakage. This is a vivid demonstration that the La–PS binding complex and the nature of the fusion intermediate are quite different in these two pH ranges.

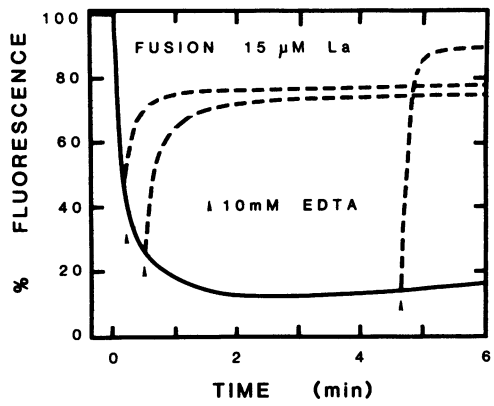


FIGURE 7 Fusion of PS LUV induced by 15 $\mu$M $La^{3+}$ at pH 7.4 and the disruption caused by adding 10 mM EDTA at the indicated times. If fusion is first stopped by addition of 1 mM $La^{3+}$ and then excess EDTA is added, there is essentially no destabilization. These data indicate that with $La^{3+}$ at neutral pH, stable intermembrane intermediates exist between apposed membranes, which are disrupted by EDTA in a fashion leading to leakage of contents.

## DISCUSSION

We have developed a direct correlation between a cation-PS binding complex and the fusion of PS LUV induced by this cation. The extremely large binding constant of $La^{3+}$ to PS, and its sensitivity to pH, imply a probable molecular structure for that binding (Fig. 8). The control of the fusion event by the electrostatic surface potential, via $La^{3+}$ binding, clearly shows that whereas the membranes need not be neutral to allow close apposition, neither can the potential exceed ~30 mV in magnitude in this electrolyte. We have proposed that while the liposome fusion rate may depend partially upon overcoming the hydration barrier, with $La^{3+}$ and PS LUV the intermembrane intermediate (or defect) between the apposed outer monolayers of the liposomes has a long lifetime at pH 7.4. If fusion occurred immediately after the apposed membranes surmounted the hydration barrier, then it would be difficult to understand how EDTA chelation could cause disruption of the fused structures.

## Binding of $La^{3+}$ to Phosphatidylserine

Using the microelectrophoretic techniques applied here, McLaughlin et al. (1981) fitted binding constants of a wide variety of divalent cations to PS, e.g., 8 $M^{-1}$ for $Mg^{2+}$, 12 $M^{-1}$ for $Ca^{2+}$, 25 $M^{-1}$ for $Mn^{2+}$ and 40 $M^{-1}$ for $Ni^{2+}$. These binding constants are quite small compared to $10^5$ $M^{-1}$ for $La^{3+}$ at pH 7.4 or even 4,000 $M^{-1}$ for $La^{3+}$ at pH 4.5. This latter value is quite similar to the carboxyl-$H^+$ binding constant of 3,000 $M^{-1}$ (Ohki and Kurland, 1981; Tsui et al., 1986).

Our experiments, as well as those of others (Hammoudah et al., 1981) show that at pH 7.4 the binding of $La^{3+}$ to PS causes the release of a proton. Sigel and Martin (1982) have reviewed the binding of certain divalent transition metals, e.g., Cu(II) and Ni(II), to serinelike molecules and concluded that the five-member ring formed by the chelation of the metal ion by the amino and carboxyl moities is common. They also concluded that $Ca^{2+}$, and probably the other alkaline-earth metals, cannot create this complex.

In Fig. 8 we propose a sequence of binding reactions for $La^{3+}$. This model presumes that the high-affinity binding between $La^{3+}$ and PS does not involve the phosphate group directly. A binding between $La^{3+}$ and the phosphate of phosphatidylcholine is known from $^{31}$P-NMR studies

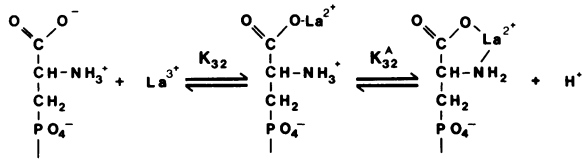


FIGURE 8 Proposed model of $La^{3+}$ binding to the PS headgroup. In the first step, the La-carboxyl group binding establishes the complex. Then an inner chelation complex forms, with La bridging between the amino and carboxyl groups. This second step requires the release of a proton, which has been demonstrated. It is not known whether the two groups must be on the same molecule, as shown here, or whether they could be on adjacent PS molecules. Some interaction with the phosphate is likely. The binding constant $K_{32}$ refers to trivalent cation binding to the second site on PS, i.e., carboxyl/amine. The first site is the $PO_4^-$ group, hence the binding constant for $Ca^{2+}$ is $K_{21}$ and for $Na^+$ is $K_{11}$. If $Mn^{2+}$ binds to the second site, we would denote its binding constant as $K_{22}$. See the Appendix.

(Hauser et al., 1977; Grasdalen et al., 1977; Westman and Erikkson, 1979). However, this binding is relatively weak and we would not detect it in the PS system. Our data implies that $Ca^{2+}$ and $La^{3+}$ bind to different sites, and Casal et al. (1987*a* and *b*) have found that $Ca^{2+}$ interacts only with the phosphate group for DMPS, POPS, and DOPS. It must be noted that Prados et al. (1974) found that lanthanide binding to amino acids at neutral pH was interfered with by hydrolysis.

Whereas this model may be improved, it captures a very useful concept. The amino group of PS normally has a $pK_a \simeq 10$ (Papahadjopoulos, 1968; MacDonald et al., 1976; van Dijck et al., 1978; Cevc et al., 1981). However, in the presence of certain transition metals, e.g., $Mn^{2+}$ and $La^{3+}$, a proton is released and the effective $pK_a$ of the amino group is reduced. If we take the effective binding constant of $La^{3+}$ to be $10^5\ M^{-1}$ at pH 7.4 and $4 \times 10^3\ M^{-1}$ at pH 4.5, then applying the analysis shown in the Appendix to the model given in Fig. 8 predicts $K_{32} = 3.9 \times 10^3\ M^{-1}$ and $K_{32}^{A} = 2 \times 10^{-6}$ M. Thus, the $La^{3+}$ chelation reduces the effective $pK_a$ to 5.7.

In Table II, we show calculated values of the surface potential and the amounts of bound $La^{3+}$ and $Ca^{2+}$ from this model. In addition, we show (in parentheses) the values when there is simple competitive (1:1) binding among all species (as prescribed in Eq. 2), taking $K_{31}$ (La) $= 10^5\ M^{-1}$. The surface potentials, and therefore the zeta potentials, are quite similar. Either model could be used to fit the zeta potential data in Fig. 4. With microelectrophoresis alone it would be difficult to distinguish between these two models. However, the low pH behavior of PS and $La^{3+}$ is much simpler to understand with the model shown in Fig. 8.[1]

A second point illustrated by this table is the amount of bound $Ca^{2+}$ one can expect in the presence of $La^{3+}$. Fig. 6, *A* and *B*, shows that the fusion is dominated by $La^{3+}$, even in 2 $\mu$M $La^{3+}$ and 5 mM $Ca^{2+}$, where the $La^{3+}$ alone is insufficient to induce rapid aggregation. The table clearly shows that the amount of bound $Ca^{2+}$ in these cases is well below its fusion threshold level of ~0.15 Ca bound/PS (Bentz and Düzgüneş, 1985). Note that the calculations in the table refer to free cation concentrations. The fusion experiments with 2 $\mu$M $La^{3+}$ include 10 $\mu$M PS, so the free concentration of $La^{3+}$ is even less than 2 $\mu$M. A rough calculation for 2 $\mu$M total $La^{3+}$, assuming 5 $\mu$M PS on the exterior surface, gives a free concentration of 0.2 $\mu$M $La^{3+}$. This case is shown in the table: 0.2 $\mu$M free $La^{3+}$ + (0.36 La bound/PS) (5 $\mu$M PS) = 2 $\mu$M total $La^{3+}$. This calculation also explains the very slow aggregation of the liposomes in 2 $\mu$M total $La^{3+}$. The predicted surface potential for 0.2 $\mu$M free $La^{3+}$ is ~−25 mV. With 5 $\mu$M total $La^{3+}$, there would be 3 $\mu$M free $La^{3+}$ and the amount of bound $Ca^{2+}$ is insignificant.

TABLE II
CALCULATED AMOUNTS OF BOUND CATIONS

| pH | $[La^{3+}]$ | $[Ca^{2+}]$ | Surface potential* | Amounts bound* La/PS | Amounts bound* Ca/PS |
|---|---|---|---|---|---|
| | *M* | *M* | *mV* | | |
| 7.4 | 0 | 0 | −76.1 (−76.1) | 0 (0) | 0 (0) |
| | 0 | $5 \times 10^{-3}$ | −32.3 (−32.3) | 0 (0) | 0.35 (0.35) |
| | $2 \times 10^{-7}$ | 0 | −22.7 (−26.2) | 0.36 (0.25) | 0 (0) |
| | $1 \times 10^{-6}$ | 0 | −10.8 (−13.2) | 0.41 (0.29) | 0 (0) |
| | $1 \times 10^{-6}$ | $5 \times 10^{-3}$ | −8.0 (−11.7) | 0.34 (0.23) | 0.09 (0.09) |
| | $2 \times 10^{-6}$ | $5 \times 10^{-3}$ | −3.5 (−6.6) | 0.38 (0.26) | 0.07 (0.06) |
| | $1 \times 10^{-3}$ | $5 \times 10^{-3}$ | +43.1 (+43.3) | 0.57 (0.38) | 0.002 (0.001) |
| 4.5 | $1 \times 10^{-6}$ | 0 | −35.2 (−38.9) | 0.20 (0.21) | 0 (0) |
| | $1 \times 10^{-6}$ | $5 \times 10^{-3}$ | −25.8 (−28.7) | 0.08 (0.06) | 0.26 (0.28) |
| | $5 \times 10^{-6}$ | $5 \times 10^{-3}$ | −18.5 (−22.0) | 0.15 (0.14) | 0.17 (0.18) |
| | $1 \times 10^{-3}$ | $5 \times 10^{-3}$ | +18.0 (+17.1) | 0.34 (0.34) | 0.01 (0.01) |
| | $2 \times 10^{-3}$ | 0 | +23.3 (+22.7) | 0.35 (0.35) | 0 (0) |

*These calculated values of surface potentials and amounts of bound cations were determined by use of the equations shown in the Appendix. The electrolyte is 0.1 M NaCl plus the cations indicated. The values in parentheses were calculated by assuming all cations compete for the same site with $K_{11}(Na^+) = 1\ M^{-1}$, $K_{21}(Ca^{2+}) = 12\ M^{-1}$, and $K_{31}(La^{3+}) = 10^5\ M^{-1}$ at pH 7.4 and 4,000 $M^{-1}$ at pH 4.5. The other values, to the left of the parentheses, were calculated assuming noncompetitive binding as described in the Appendix. $La^{3+}$ was assumed to bind only to the $S_2$ site (carboxyl/amine) with a binding constant to the carboxyl of $K_{32} = 3.9 \times 10^3\ M^{-1}$ and an amine chelation dissociation constant of $K_{32}^{A} = 2 \times 10^{-6}$ M. This corresponds to the amine group having a $pK_a = 5.7$ in the presence of $La^{3+}$. $Ca^{2+}$ and $Na^+$ were assumed to bind to the $S_1$ site (phosphate) with the same binding constants used for the competitive binding. The actual site of $Na^+$ binding is problematical, but for the purpose of this calculation, it makes no significant difference whether it is assigned to the phosphate or to the carboxyl group. In previous work $Ca^{2+}$ and $Na^+$ were assumed to compete for binding sites (Nir et al., 1978; McLaughlin et al., 1981). However, Cohen and Cohen (1981) showed that the experimental data might also be described by assigning the cations to noncompeting binding sites. The temperature was 25°C and the other parameters are defined in the Appendix.

In previous studies on $La^{3+}$/PS interactions, Ohki and Duax (1986) found that 15–20 $\mu$M $La^{3+}$ was required to induce the fusion of PS SUV to PS monolayers as monitored by a decrease in the monolayer surface tension. They also found surface charge neutrality of PS MLV at 150 $\mu$M $La^{3+}$, using microelectrophoresis. This is about an order of magnitude larger than the value we report here. Hammoudah et al. (1981) used atomic absorption spectroscopy to measure the amounts of $La^{3+}$ associated with PS SUV and obtained binding constants several orders of magnitude less than those reported here. The source of this discrepancy is not known. Deleers et al. (1985) have studied the interaction between $Al^{3+}$ and PS SUV and

[1]We have also considered the case where all cations compete for the same site plus the additional chelation site for $La^{3+}$, i.e., for $Na^+$, $K_{12} = 1\ M^{-1}$, for $Ca^{2+}$, $K_{22} = 12\ M^{-1}$, and for $La^{3+}$, $K_{32} = 3{,}900\ M^{-1}$ and $K_{32}^{A} = 2 \times 10^{-6}$ M. In this event, the calculated surface potentials are essentially identical to the values in parentheses and the calculated amounts of bound cations are essentially identical to those of the noncompetitive binding model. Thus, all of the binding configurations yield the same electrostatic predictions due to the different fitted binding constants.

found an aggregation and leakage threshold of ~90 $\mu$M. Jones et al. (1986) reported little or no contents mixing (using the Tb/DPA assay) with PS SUV from 1 $\mu$M–3 mM $La^{3+}$. If there was membrane destabilization here, rapid influx of the $La^{3+}$ ions would prevent $Tb(DPA)_3^{3-}$ complexation. This has been found with $Ni^{2+}$- and $Mn^{2+}$-induced fusion of PS LUV (Bentz and Düzgüneş, 1985).

## Fusion Induced by $La^{3+}$

The most obvious difference between $Ca^{2+}$- and $La^{3+}$-induced fusion of PS liposomes is that the fusion with $La^{3+}$ is nonleaky after extensive fusion. The kinetic analysis of $Ca^{2+}$-induced fusion of PS LUV showed that the initial round of fusion occurs without leakage (Wilschut et al., 1980, 1981, 1983, 1985*b*; Nir et al., 1982; Bentz et al., 1983*a*, 1985*a*). The observed loss of contents follows from subsequent aggregation, fusion, and collapse of the fused structures. This may be contrasted with the $Ca^{2+}$-induced fusion of PS SUV which incurs about 6–10% loss of contents per fusion event, in addition to the effect of the higher order aggregate/fusion products (Wilschut et al., 1980; Nir et al., 1980; Bentz et al., 1983*a*).

The detailed kinetic analysis of PS LUV fusion induced by $Ca^{2+}$, $Ba^{2+}$, and $Sr^{2+}$ showed that leakage could simply demark the collapse of the fusion products. The rate of collapse was at least 50 times smaller than the fusion rate (Bentz et al., 1983*a*, 1985*a*). This mechanism of leakage was proposed by Wilschut et al. (1980, 1981) and recently visualized by Rand et al. (1985) and Kachar et al. (1986) using rapid freeze-fracture electron microscopy. Whereas Rand et al. (1985) observed relatively fast collapse processes relative to the leakage of contents observed in this study, their result was due to the much larger lipid concentrations (>1 mM) (see also Miller and Dahl, 1982; Nir et al., 1982) and the very low $Na^+$ concentrations (2 mM) which incur very large amounts of bound $Ca^{2+}$ (Düzgüneş et al., 1981; Bentz et al., 1983*a*). Both effects accelerate the overall rate of fusion and, therefore, the subsequent collapse (Düzgüneş and Bentz, 1988). The $Ca^{2+}$-induced rupture of the very large ($\geq$10 $\mu$m) multilamellar, and multivesicular, PS liposomes observed by Rand et al. (1985) and Kachar et al. (1986) using video-enhanced light microscopy is not a process found with the LUV, at least under conditions (e.g., ionic strength) where the fluorometric assays of leakage and mixing of aqueous contents can be used.

## Mechanisms of Fusion

Within the transformation of aggregated dimer to fused doublet,

$$V_2 \xrightarrow{f_{11}} F_2,$$

we can conjecture several discrete steps. The first of these steps is the thinning of the aqueous layer between the membrane surfaces (Le Neveu et al., 1976, 1977; Cowley et al., 1978; Parsegian et al., 1979; Lis et al., 1981 *a* and *b*, 1982; Rand, 1981; Loosley-Millman et al., 1982; Rand and Parsegian, 1984, 1986; Marra, 1985, 1986; Marra and Israelachvili, 1985; Rand et al., 1985; Evans and Parsegian, 1983, 1986; Evans and Needham, 1986). This thinning is tantamount to the membranes' overcoming the hydration force between them. With the very large liposomes, Rand et al. (1985), Evans and Needham (1986), and Kachar et al. (1986) have shown that contact leads to a widening area of adhesion. A large enough area of contact can produce sufficient stress on the membranes to induce rupture, lysis, or fusion.

Nevertheless, optical studies cannot show what happens between the apposed membranes before the fusion event. The fluorometric assays based upon lipid mixing, leakage, and contents mixing provide some information. There is now clear evidence that under the proper conditions some liposomes, including PS with $Ca^{2+}$, show lipid mixing well before contents mixing, and there are even examples of lipid mixing without leakage or mixing of contents (Rosenberg et al., 1983; Ababei and Hildenbrand, 1984; Bondeson and Sundler, 1985; Ellens et al., 1985; Wilschut et al., 1983, 1985*a* and *b*; Leventis et al., 1986; Düzgüneş et al., 1987*a*). Therefore, we can dissect the path from $V_2$ to $F_2$ into three distinct stages (Düzgüneş and Bentz, 1988; Bentz and Ellens, 1988): (*a*) contact and adhesion, which is slow with the very large liposomes and may be further dissected; (*b*) formation of a defect in the outer (apposed) monolayers leading to some exchange of their lipids, known as semi- or hemifusion of the membranes; (*c*) penetration of the defect into the inner monolayers where there is a rapid propagation of morphological changes leading to the fused doublet, $F_2$. The value of $f_{11}$ will reflect the relative rates of these three events, when $f_{11}$ is determined by a contents mixing assay. These steps are illustrated in Fig. 9.

Two very important questions regarding the molecular mechanism of fusion are which of these steps is rate limiting, and why do some liposomes fuse, i.e., mix aqueous contents, and others show fusion with leakage or simply lysis. That is, surmounting the hydration force at step *a* may be rate limiting to the overall fusion event in some cases, but the question about fusion vs. lysis depends upon the nature of the defect at step *b*.

Analysis of fusion mechanisms based upon just the first step, i.e., adhesion and breakdown of the hydration force, has led to the proposal that when the membrane tension exceeds some critical value, the liposomes either fuse or lyse, depending upon whether the rupture occurs in the area of contact or not (Rand and Parsegian, 1984, 1986; Rand et al., 1985; Evans and Needham, 1987; Kachar et al., 1986). That is, some dimers fuse, whereas others lyse.

Ohki (1982, 1984) has proposed that PS liposome fusion will occur only if a monolayer of PS at an air water

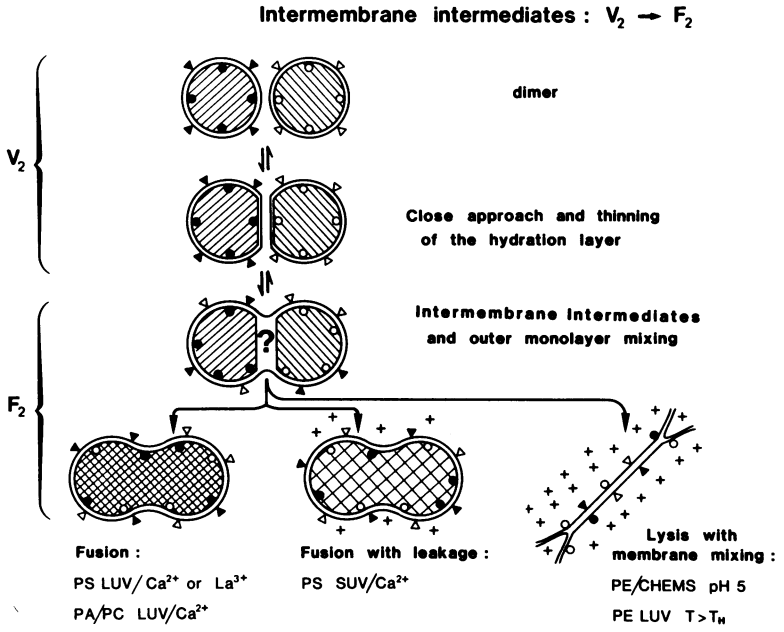


FIGURE 9 Proposed structures of initial fusion events where (*a*) the close apposition of the dimer leads to (*b*) the formation of stable intermembrane intermediates. With $La^{3+}$, these intermembrane intermediates lead to (*c*) nonleaky fusion and are stable at pH 7.4 and transient at pH 4.5. For other cases, the intermembrane intermediates could promote either fusion with some leakage or a straightforward lysis of the liposomes. The nature of the cation–lipid complex determines the type of intermembrane intermediate and outcome with respect to fusion. Prototypical cases are noted beneath each outcome. $T_H$ for PE-containing liposomes refers to the temperature of the $L_\alpha$-$H_{II}$ phase transition for the bulk lipid. Additional discussion can be found in Bentz and Ellens (1988) and Düzgüneş and Bentz (1988).

interface undergoes a sufficiently large increase in surface tension (at a fixed area) when exposed to the fusogenic cations in the subphase. This model provides a possible mechanical explanation for membrane rupture after adhesion. The number of correlations presented in Ohki (1982, 1984), Ohki and Ohshima (1984, 1985) and Ohki and Duax (1986) to support this model is large, but in the experimental systems reported, it has not been determined whether the initial rate of aqueous contents mixing is dominated by the rate of aggregation or the rate of fusion. Thus, the change in monolayer surface tension may be correlated with either the rate constant of aggregation, $C_{11}$, or the fusion rate constant, $f_{11}$. Secondly, in Ohki (1982) it was shown that in 500 mM NaCl and up to 10 mM $Ca^{2+}$, the monolayer surface tension change was less than the prescribed threshold for fusion and that there was no observed fusion of PS SUV. This was taken as a confirmation of the model. In Bentz et al. (1983*b*), the same system showed extensive fusion under fusion rate-limiting kinetics.

The most important point is that fusion mechanisms based solely upon adhesion, breakdown of the hydration force, or surface tension changes cannot explain the observation that rupture almost always occurs within the area of contact. The nonleaky fusion of PS LUV by $La^{3+}$ must imply that rupture always occurs within the area of contact. The same is true for the $Ca^{2+}$-induced fusion of the PS LUV, because the initial fusion is nonleaky. In fact, Rand and Parsegian (1986) have calculated that the giant PS liposomes in $Ca^{2+}$ have a probability of rupture per unit area that is about 10 times greater within the area of contact than outside it. The observed rupture of the giant PS liposomes outside of the contact area may reflect a radial dependence of mechanical stability (Evans and Parsegian, 1983).

## Intermembrane Intermediates

The burst of leakage from the PS LUV fusing in $La^{3+}$ at pH 7.4 after addition of EDTA, Fig. 7, is quite different from the rapid cessation of fusion observed when the pH is low or when $Ca^{2+}$ is the fusogenic cation (Figs. 1 and 5). This behavior is quite difficult to explain if the liposomes in these cases cannot spend some time in step (*b*) of Fig. 9. Pulling the fusogenic ions off the headgroups in steps (*a*) or (*c*) should incur no additional fusion or leakage. At step (*b*), the removal of the fusogenic cations would require the doublets to choose between either completing the process or breaking off the connection.

Actually, the lysis we observed after EDTA addition at pH 7.4 is relatively uncommon and it is very interesting that the $Ca^{2+}$-induced fusion of DOPA/DOPC LUV is nearly identical. The fusion is nonleaky and EDTA addition induces lysis (Leventis et al., 1986). What makes this significant is that Smaal et al. (1987) have found that DOPA/DOPC and $Ca^{2+}$ produce NMR resonances indicative of immobilized headgroups and very mobile acyl chains. More detailed analysis is needed, but it is encouraging that this system shows an unusual structure. Obviously, a similar analysis for La/PS is needed.

The correlation between the structure of the $La^{3+}$-PS binding complex and the stability of the intermembrane intermediates at step (*b*) is quite provocative. At pH 7.4, $La^{3+}$ is chelated to both PS sites, the fusion is extensive and nonleaky, the intermembrane intermediates are long-lived, and the removal of the $La^{3+}$ by EDTA at this stage causes these structures to lyse. Addition of excess $La^{3+}$ results in a nonleaky separation of the membranes. At pH 4.5, the $La^{3+}$ is bound only to one site, the fusion is still nonleaky, but the intermembrane intermediates are sufficiently short-lived that removing $La^{3+}$ by EDTA produces no extra leakage.

We have applied this type of analysis to phosphatidylethanolamine-containing liposomes and found that there are at least two mechanisms leading to mixing of aqueous contents, one operating when the lipid (in bulk phase) is in the $L_\alpha$ phase and one operating when the lipid (in bulk phase) shows isotropic $^{31}$P-NMR resonances (Ellens et al., 1986*b*). When the lipid can transform ultimately to the $H_{II}$ phase, there is a rapid contact-mediated lysis of the liposomes (Bentz et al., 1985*b*, 1987; Ellens et al., 1986*a* and *b*). The mechanism operating in the temperature range of the isotropic state, i.e., when the $^{31}$P-NMR shows isotropic resonances, appears to imply that the defects at step (*b*) either are, or can transform to, structures which cause communication of the internal contents, i.e., fusion (Ellens et al., 1986*b*; Siegel, 1986*a* and *b*, 1987).

## Concluding Remarks

Comparison of $La^{3+}$- and $Ca^{2+}$-induced fusion of PS liposomes has produced a coherent framework for examining the molecular mechanisms of liposome and biological membrane fusion. We emphasize the importance of both the rate-limiting step of the fusion process and the structure of the intermembrane intermediate or defect in the area of contact which will determine the extent to which mixing of internal contents will occur. Overcoming the hydration barrier and formation of the intermembrane intermediate are independent kinetic steps. Evidence from a wide range of lipid compositions indicates instances where these two steps clearly work at cross purposes with respect to fusion. In this regard, the fusion mechanism is a balance of average surface forces, which may tend to promote lysis, and the intermembrane intermediates, which arise from defects in these surfaces within the area of contact and which may or may not promote fusion. The fusion induced by $La^{3+}$ is nonleaky regardless of whether the $La^{3+}$ is bound to two sites on PS, at pH 7.4, or one site, at pH 4.5. However, the stability, or lifetime, of the intermembrane intermediate is clearly longer when both sites are bound.

## APPENDIX

Here we will briefly sketch the equations used for the theoretical calculations of zeta potentials and amounts of bound cations used in the text. More details can be found in Bentz (1981, 1982) and Cohen and Cohen (1981). The PS headgroup can be considered as a pair of binding sites denoted $S_1^-$ $S_2$, where $S_1^-$ is the phosphate group and $S_2$ is $-CH_2CH(NH_3^+)COO^-$. We presume here that the sites are independent, thus, if $C_j^{j+}$ denotes the *j*-valent cation, we can write the binding reactions as

$$C_j^{j+} + S_1^- \overset{K_{j1}}{\rightleftharpoons} C_jS_1^{(j-1)+},$$

$$C_j^{j+} + S_2 \overset{K_{j2}}{\rightleftharpoons} C_jS_2^{j+} \overset{K_{j2}^A}{\rightleftharpoons} C_jS_2^{(j-1)+} + H^+.$$

If $\sigma_o$ and $\sigma$ denote the surface charge densities before and after cation binding to the sites, then

$$\frac{\sigma}{\sigma_o} = 1 - \frac{\sum_1^3 jK_{j1}C_j(\infty)y_o^j}{1 + \sum_j^3 K_{j1}C_j(\infty)y_o^j} - \frac{\sum_j^3 jK_{j2}\left(1 + \frac{j-1}{j}\frac{K_{j2}^A}{[H^+]}\right)C_j(\infty)y_o^j}{1 + \sum_j^3 K_{j2}\left(1 + \frac{K_{j2}^A}{[H^+]}\right)C_j(\infty)y_o^j}, \quad \text{(A1)}$$

where $C_j(\infty)$ is the bulk concentration of the *j*-valent cation and $y_o = \exp\{-e\psi(o)/kT\}$ is the Boltzmann factor with $\psi(o)$ being the electrostatic surface potential. The value of $y_o$ is the solution to

$$0 = \left[G\frac{\sigma}{\sigma_o}\right]^2 - \Delta\left|(y_o^{-1} - 1) + \sum_j^3 c_j(y_o^j - 1)\right|, \quad \text{(A2)}$$

where $\Delta = \text{sgn}(\sigma/\sigma_o)$, and

$$c_j = C_j(\infty)/\gamma^2,$$

$$\gamma^2 = \sum_j^3 jC_j(\infty),$$

$$G = \frac{271.85\omega(T)}{a\gamma},$$

$$\omega(T) = (80.4)(293.15)/\epsilon T. \quad \text{(A3)}$$

$a$ is the area (Å$^2$) per charge before binding (70Å$^2$ for PS); $\epsilon$ is the dielectric constant of the medium ($\epsilon = 78.5$ at 25°C); $T$ is the absolute temperature. It is assumed that there are only monovalent anions which do not bind. This equation also assumes that the liposomes are sufficiently large to be treated as flat plates. Corrections for the effect of radius can be found in Bentz (1981, 1982) and Ohshima et al. (1982).

The amount of the *j*-valent cation bound per PS headgroup is given by

$$RB_j = \frac{K_{j1}C_j(\infty)y_o^j}{1 + \sum_i^3 K_{i1}C_i(\infty)y_o^i} + \frac{K_{j2}\left(1 + \frac{K_{j2}^A}{[H^+]}\right)C_j(\infty)y_o^j}{1 + \sum_i^3 K_{i2}\left(1 + \frac{K_{i2}^A}{[H^+]}\right)C_i(\infty)y_o^i}. \quad \text{(A4)}$$

We estimate the zeta potential using an approximate solution to the Poisson–Boltzmann equation developed in Bentz (1982) as

$$\psi(r) = -\frac{kT}{e}\ln\left[\frac{(\theta+1)^2}{(\theta-1)^2 - 4\theta(\alpha + c_3y_o)}\right], \quad \text{(A5)}$$

where $kT/e = 25.69$ mV at 25°C and

$$\alpha = 2c_3 + c_2,$$

$$\theta = A(y_o)\exp(\lambda\kappa r),$$

$$A(y) = [2t(y)t(1) + 2(\alpha + c_3y_o)y + y + 1]/(y-1),$$

$$t(y) = [(c_3y_o + \alpha)y^2 + y]^{1/2},$$

$$\lambda^2 = 1 + c_3[(y_o+1)^{1/2} - 1]/(c_3 + \alpha + 1),$$

$$\kappa = 0.3274\eta\gamma\omega(T)^{1/2}\,\text{Å}^{-1},$$

$$\eta = (1 + c^2 + 3c_3)^{1/2} \quad \text{(A6)}$$

and $r$(Å) is the distance from the surface.

We have found a useful rough solution for $y_o$ in Eq. A2 to be given by

$$y_o \simeq \left[K_{32}\left(2 + \frac{K_{32}^A}{[H^+]}\right)C_3(\infty)\right]^{-1/3}, \qquad \text{(A7)}$$

which is asymptotically valid when $y_o \to 1$. Comparison of the estimates given by Eq. A7 and the exact values shown in Table II show its usefulness and range of validity.

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