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Phospholipid Vesicle Aggregation: Effect of Monovalent and Divalent Ions†

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ABSTRACT: A study on cation-induced aggregation of unilamellar phospholipid vesicles was made by measuring the turbidity of vesicle suspensions. As the cation concentration was increased, the degree of aggregation of acidic phospholipid vesicles increased. The concentration at which the sharpest increase in turbidity was observed was defined as the "threshold" concentration. The threshold concentrations of various divalent cations for aggregation of phosphatidylserine vesicles in 0.1 M NaCl, pH 7.0, were 0.65 mM Mn^{2+}, 0.8 mM Ba^{2+}, 1.0 mM Ca^{2+}, 1.2 mM Sr^{2+}, and 4.5 mM Mg^{2+}. This order of threshold concentrations was the same for vesicles composed of mixtures of phosphatidylserine and phosphatidylcholine. A decrease in the monovalent ionic strength of the vesicle suspension solutions raised the threshold concentrations for Mn^{2+} and Ca^{2+}. The order of effectiveness for monovalent cations to cause phosphatidylserine vesicle aggregation was $H^+ > Na^+ > Li^+ > K^+ > TMA^+$ (tetramethylammonium). The effect of pH on phosphatidylserine vesicle aggregation was studied in the presence of Na^+. The threshold pH for vesicle aggregation was about 2.6 in 100 mM NaCl. An attempt was made to explain vesicle aggregation in terms of surface potential, surface charge densities of the membranes, and the electrostatic repulsive interaction energy between two vesicles. It was proposed that the slope of the total interaction energy (repulsive interaction) at the Debye distance is related to the degree of massive aggregation occurred among phosphatidylserine vesicles. Knowing the intrinsic binding constants of Ca^{2+} (30 M^{-1}) and Na^+ (0.6 M^{-1}) to a phosphatidylserine membrane and assuming that the slope of the interaction energy of two interacting membranes is the same at the Debye distance at the observed threshold concentrations of ions for vesicle aggregation, we calculated the binding constants of other metal ions: Mn^{2+} = 49 M^{-1}, Ba^{2+} = 37 M^{-1}, Sr^{2+} = 25 M^{-1}, Mg^{2+} = 10 M^{-1}, Li^+ = 0.4 M^{-1}, K^+ = 0.2 M^{-1}, and H^+ = 100 M^{-1}.

For elucidation of the molecular mechanism of membrane fusion, which is involved in many biological cellular processes (Poste & Allison, 1973), membrane fusion studies using lipid model membrane systems have recently been made by a number of laboratories (Prestegard & Fellmeth, 1974; Papahadjopoulos et al., 1974; Breisblatt & Ohki, 1975; Koter et al., 1978; Ingolia & Koshland, 1978; Liao & Prestegard, 1979; Wilschut & Papahadjopoulos, 1979). The close contact of two membranes is considered to be an initial step and a necessary condition for two membranes to fuse. Thus, cell membrane aggregation studies should be relevant for understanding conditions and control of membrane fusion reactions. For vesicle aggregation studies, turbidity (Chong & Colbow, 1976) and light scattering (Lansman & Haynes, 1975; Day et al., 1977) methods have been frequently utilized. Although the interpretation of turbidity is not as clear as that of light scattering, turbidity studies have provided some useful information for elucidation of the mechanisms of aggregation, fusion, and membrane structural alterations of vesicle membranes.

The effect of temperature on the turbidity of lipid vesicle suspensions has been studied in relation to the phenomenon of the gel to liquid-crystalline phase transition (Yi & MacDonald, 1973; Martin & MacDonald, 1976; Peterson & Chan, 1978; Kremer & Wiersema, 1977). It is well documented that acidic phospholipid vesicles aggregate in the presence of cations whose "threshold" concentration depends upon the membrane composition and concentrations of ionic species in the suspension solution. The flocculation of sonicated phosphatidylserine vesicles due to high concentrations of NaCl and KCl and millimolar concentrations of $CaCl_2$ and $MgCl_2$ has been observed by several investigators (Abramson et al., 1964; Hauser & Phillips, 1973; Papahadjopoulos et al., 1975). The irreversible changes in flocculation have been interpreted as evidence for membrane fusion and structural alterations of vesicle membranes (Papahadjopoulos et al., 1974; Chong & Colbow, 1976). However, further systematic studies of phospholipid vesicle aggregation are necessary to understand ionic and membranous factors that control membrane fusion reactions. These factors include the surface charge density

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and lipid composition of the vesicle membranes (also proteins associating with membranes) and various monovalent and divalent ion concentrations in the vesicle suspension media.

Here, for examination of these factors, a lipid vesicle aggregation study was performed with variations in lipid components (phosphatidylserine and phosphatidylcholine), monovalent (H^+, Na^+, K^+, Li^+, and TMA^+)[1] and divalent (Mn^{2+}, Ba^{2+}, Ca^{2+}, Sr^{2+}, and Mg^{2+}) ion concentrations, and their ionic strength. The results are compared in terms of the surface potential, surface charge density of the membranes, and the electrostatic energy acting between two vesicle membranes.

Materials and Methods

Chemicals. Bovine phosphatidylserine (PS) and egg phosphatidylcholine (PC) were purchased from Avanti Biochemical Co. Both samples showed a single spot on silica gel thin-layer chromatographic plates. Chloride salts used were of reagent grade purchased from Fisher Chemical Co. Lipid vesicle suspension solutions were either NaCl or TMA-Cl, containing small amounts of Hepes [*N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Calbiochem] or sodium citrate (Fisher Scientific Co.) as buffer, and 0.01 mM EDTA (Fisher Chemical Co.). The pH of the solution was adjusted with either NaOH or HCl. The water used was distilled 3 times, including the process of alkaline permanganate.

Vesicle Preparation. Unilamellar phospholipid vesicles were prepared according to the published method (Düzgüneş & Ohki, 1977). Phospholipids were suspended at a concentration of 10 μmol of phospholipid/mL in a buffered salt solution, vortexed for 10 min, and sonicated for 1 h in a bath-type sonicator (Heat Systems, Ultrasonics) at 20 to 24 °C under an N_2 atmosphere. Vesicle suspensions were centrifuged at 100000*g* for 1 h, and the supernatant was used for the experiments. The concentration of phospholipid in the suspension was determined by phosphate analysis (Fiske & Subbarow, 1925).

Turbidity Measurements. Turbidity of the vesicle suspensions as a function of divalent ion (Mn^{2+}, Ba^{2+}, Ca^{2+}, Sr^{2+}, and Mg^{2+}) and monovalent ion (Na^+, Li^+, K^+, and TMA^+) concentrations and various pHs was measured at 400 nm by use of a Hitachi (100–60) spectrophotometer. The wavelength in the visible range at which sonicated unilamellar phosphatidylserine vesicles caused maximum light scattering was found to be approximately 400 nm. For most cases the vesicles were suspended at 0.1 μmol of phospholipid/mL in the same solution used for vesicle preparation, and the ion concentrations were raised step by step by introducing small amounts of its concentrated salt solution (3 M). The absorbance was measured 2 min after changing the ion concentration, and then the concentration was increased. The procedure was repeated until the absorbance no longer changed (or began to decrease). All experiments were done at room temperature, 24 ± 2 °C. Each point in all figures shown is the average of at least five experiments.

Threshold Concentration for Vesicle Aggregation. The absorbance obtained 2 min after changing each ion concentration was plotted as a function of cation concentration. The cation concentration corresponding to the maximum increase in the rate of absorbance change was obtained from this curve and defined as the "threshold concentration" for that cation to induce vesicle aggregation. Control experiments where the threshold concentration was determined by either (1) measuring the maximum absorbance obtained at each ion concentration and then plotting the maximum increase in absorbance with respect to cation concentration or (2) plotting the initial rate of absorption intensity increase against each ion concentration gave the same results (Düzgüneş, 1978). The 2-min method was chosen because of its experimental simplicity and quickness.

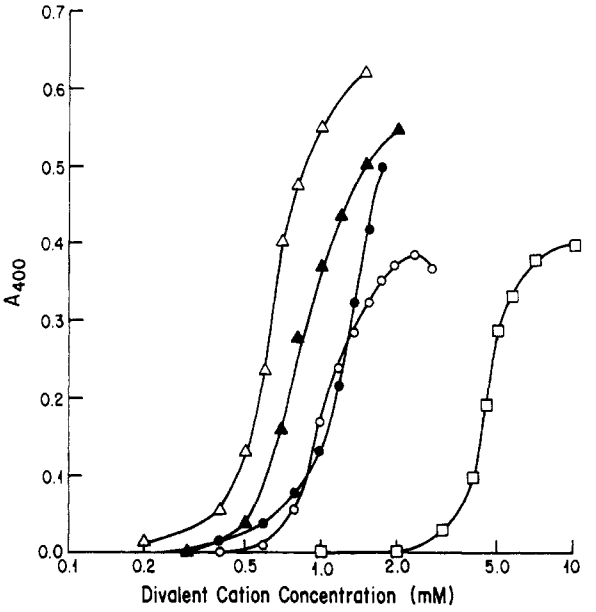

FIGURE 1: Turbidity (A_{400}) of phosphatidylserine vesicle suspensions (0.1 μmol of lipid/1 mL of 0.1 M NaCl–5 mM Hepes–0.05 mM EDTA, pH 7.0) vs. divalent cation concentrations. Turbidity at 2 min after the change in divalent ion concentration was plotted against the final divalent ion concentration. (△) Mn^{2+}; (▲) Ba^{2+}; (○) Ca^{2+}; (●) Sr^{2+}; (□) Mg^{2+}.

Results

Figure 1 shows the relation of turbidity vs. various divalent cation concentrations for PS vesicle aggregation in 0.1 M NaCl, pH 7.0. The observed decrease in absorbance for 2 mM Ca^{2+} is probably due to the formation of very large flocculates, which causes an increase in the mean free path of the light as a result of the decreased concentration of light scattering centers.

The order of effectiveness for divalent cations to induce vesicle aggregation was $Mn^{2+} > Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$. The threshold concentrations of these divalent ions (giving the sharpest increase in turbidity) were 0.65, 0.8, 1.0, 1.2, and 4.5 mM, respectively.

In order to check the reversibility of the aggregation reaction, we added EDTA at twice the concentration of divalent ions present to chelate all divalent cations in the suspension. For all divalent ions, except Mg^{2+}, when divalent ion concentrations were above the threshold concentration, the increased turbidity did not go back to the initial level by the addition of the above-mentioned amount of EDTA. Instead it was reduced to about 20 to ~25% of the intensity before the addition of EDTA. In the case of Mg^{2+}, however, the turbidity returned almost to zero after EDTA addition. These observations suggest that at or above their respective threshold concentration, Mn^{2+}, Ba^{2+}, Ca^{2+}, and Sr^{2+} caused considerably more irreversible aggregates, structural alteration of vesicles, or vesicle fusion than Mg^{2+}.

The second series of experiments was carried out to examine the effect of the content of acidic phospholipids in vesicle membranes on divalent ion induced vesicle aggregation. In Figure 2, the threshold concentrations of various divalent ions

[1] Abbreviations: TMA^+, tetramethylammonium; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

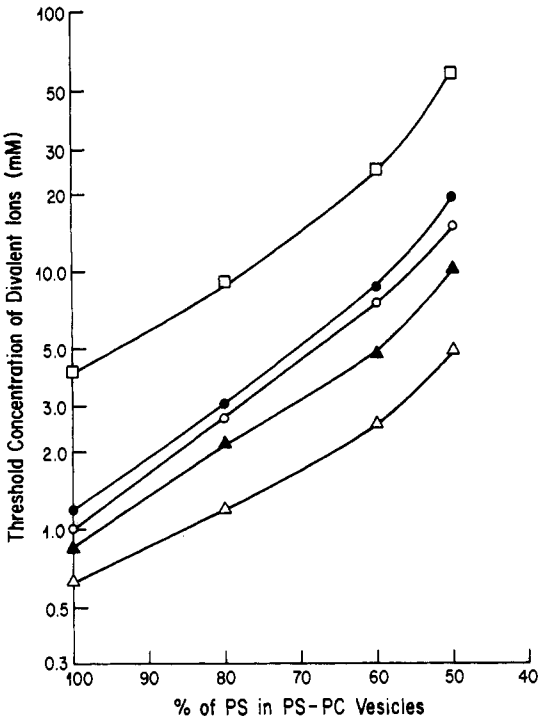

FIGURE 2: Threshold concentrations of divalent cations for aggregation of phospholipid vesicles made of different ratios of phosphatidylserine to phosphatidylcholine. The concentration of phosphatidylserine in a vesicle preparation is expressed in weight percentage. The vesicles were suspended in a 0.1 M NaCl–5 mM Hepes–0.05 mM EDTA, pH 7.0, solution at a concentration of 0.1 μmol of lipid/mL. (△) Mn^{2+}; (▲) Ba^{2+}; (○) Ca^{2+}; (●) Sr^{2+}; (□) Mg^{2+}.

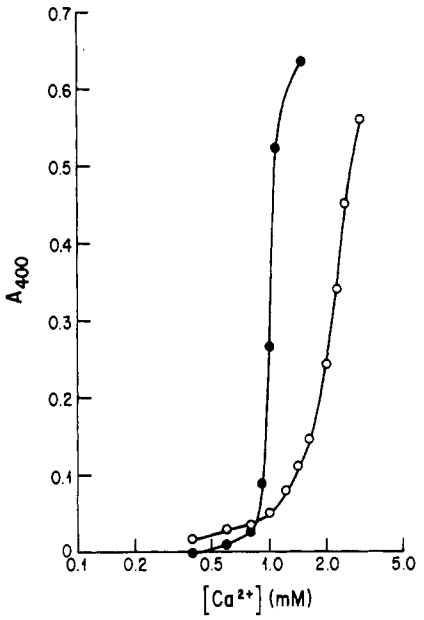

FIGURE 3: Turbidity (A_{400}) of phosphatidylserine vesicle suspension in different ionic strengths of NaCl with respect to Ca^{2+} concentrations. (●) 0.2 μmol of lipid/1 mL of 0.1 M NaCl–5 mM Hepes–0.05 mM EDTA, pH 7.0; (○) 0.2 μmol of lipid/1 mL of 0.01 M NaCl–1 mM Hepes–0.01 mM EDTA, pH 7.0.

for vesicles composed of various ratios of phosphatidylserine and phosphatidylcholine are shown. The order of the threshold concentrations observed for the divalent ions was the same for all the phosphatidylserine/phosphatidylcholine mole fractions. The smaller the amount of phosphatidylserine in the vesicle, the greater the values for the threshold concentration that were obtained. The results for phosphatidylserine/phosphatidylcholine (1:1) vesicle suspension correspond to those reported previously (Ohki & Düzgüneş, 1979).

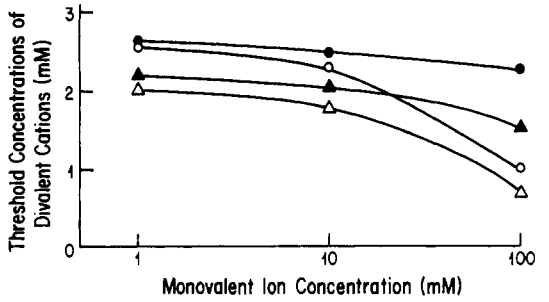

FIGURE 4: Threshold concentrations of divalent cations for aggregation of phosphatidylserine vesicles suspended in salt solutions with various ionic strengths. The salt solutions were either NaCl or TMA^+ with 5 mM Hepes–0.05 mM EDTA, pH 7.0, for the 100 mM salt case, 0.5 mM Hepes–0.01 mM EDTA, pH 7.0, for the 10 mM salt case, and 0.1 mM Hepes–0.005 mM EDTA, pH 7.0, for the 1 mM salt case. (△) NaCl + Mn^{2+}; (▲) TMA^+ + Mn^{2+}; (○) NaCl + Ca^{2+}; (●) TMA^+ + Ca^{2+}.

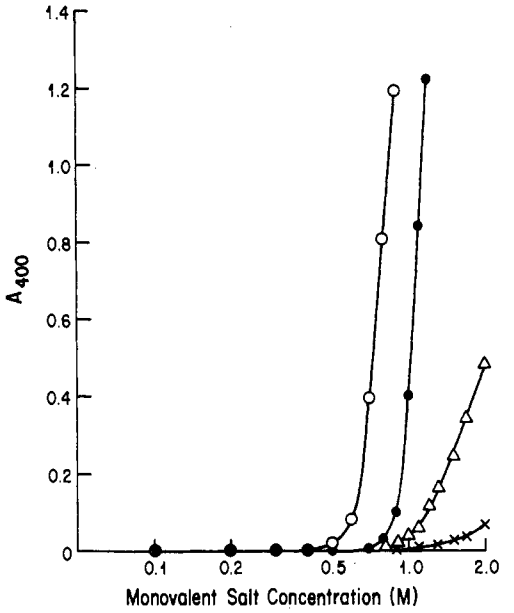

FIGURE 5: Turbidity (A_{400}) of phosphatidylserine vesicles with respect to monovalent salt concentrations. The lipid vesicle concentrations were 0.5 μmol of phosphatidylserine/1 mL of 0.1 M salt solution containing 5 mM Hepes and 0.05 mM EDTA, pH 7.0. (○) NaCl; (●) LiCl; (△) KCl; (×) TMA^+.

In the third series of experiments, the effect of monovalent ionic species and ionic strength on divalent ion induced aggregation was studied. Figure 3 shows the change in tubidity of phosphatidylserine vesicle suspensions in two ionic strengths of NaCl. The increase in absorbance began at lower concentrations of divalent ions, as the monovalent ionic strength was reduced from 0.1 to 0.01 M, indicating that the greater surface potential due to the low ionic strength of a bulk solution increases the amount of bound divalent ions and results in aggregation and possible fusion. The threshold Ca^{2+} concentration for inducing the fusion of sonicated phosphatidylserine/phosphatidylcholine or phosphatidylserine vesicles was also lower in 3 mM than in 100 mM NaCl (Düzgüneş et al., 1981). It should be noted however that the sharpest increase in absorbance in Figure 4 occurs at higher Ca^{2+} and Mn^{2+} concentrations, when the Na^+ concentration is lowered. This effect was less pronounced for TMA^+ (Figure 4). The initial increase in turbidity (Figure 3) may be due to the dimerization and fusion of the vesicles into spheres of larger diameters.

Vesicle aggregation due to monovalent cations is shown in Figure 5. The order of effectiveness of monovalent ions found

to induce PS vesicle aggregation was $Na^+ > Li^+ > K^+ > TMA^+$. The threshold concentrations obtained for the monovalent cations were 0.8 M for Na^+, 1.1 M for Li^+, and 1.5 M for K^+, though they were more difficult to determine exactly because vesicle aggregation was less sensitive to changes in monovalent cation concentration. In all cases of monovalent cation-induced vesicle aggregation, the turbidity was reduced to the reference value when the salt concentration was diluted to less than half of their threshold values, indicating that the aggregation was fully reversible and that possibly no vesicle fusion occurred. The result for Na^+ compares well with that reported earlier (Day et al., 1980; Nir et al., 1980).

The pH dependence of the turbidity of phosphatidylserine vesicle suspensions in 0.1 M NaCl solutions was also determined. No significant change was observed in the pH range 3.5–8.0. Below pH 3.0, the turbidity increased gradually as the pH of the bulk phase was reduced. It then sharply increased between pH 2.5 and 2.7. The threshold pH that induced PS vesicle aggregation in a 0.1 M NaCl solution was taken to be 2.6.

Discussion

The experimental results obtained in this study can be divided into three sections. In the first section, a base line was established for the divalent cation-induced aggregation of PS vesicles under "standard conditions". The second section consisted of measuring the effects of changing the experimental conditions on this base line. The experimental conditions were modified either by changing the concentration of PS molecules within the membrane, and thus negative charges per unit area membrane, or by altering the standard conditions, i.e., species and ionic strength of monovalent ions (NaCl or TMA^+) present in the PS vesicle suspensions. In the third section, the order of monovalent cation effectiveness and pH effects were determined.

The base-line divalent-cation experiments were conducted under the same standard conditions employed in studies of PS membrane fusion and thus can be compared to such studies. The order of divalent cation effectiveness causing PS vesicle aggregation ($Mn^{2+} > Ba^{2+} > Ca^{2+} \geq Sr^{2+} > Mg^{2+}$) found in this study, as well as their threshold concentrations, is similar to that observed to induce the fusion of PS vesicles (S. Ohki, unpublished data). This ion sequence also corresponds to that obtained for the relative inhibition of Ca^{2+} binding to PS molecules (Blaustein, 1967) as well as that which induces the fusion of PS vesicles to a PS monolayer (Ohki & Düzgüneş, 1979). The threshold concentration obtained for Mg^{2+}-induced PS vesicle aggregation in this study (4.5 mM) was, however, lower than that reported to induce PS vesicle fusion (6 mM) by Wilschut et al. (1981). This difference is consistent with the results obtained in the EDTA experiments, where EDTA addition resulted in only a partial reversal of PS vesicle aggregation caused by Mn^{2+}, Ba^{2+}, Ca^{2+}, or Sr^{2+} (possible fusion) but in an almost total reversal of Mg^{2+}-induced PS vesicle aggregation, suggesting that no fusion occurs at this concentration.

The results obtained in the experiments where the PS content within a membrane was modified indicate that divalent cation-induced vesicle aggregation does vary with the quantity of acidic sites per unit area of membrane and the degree of ion binding to polar groups of the phospholipid molecule (Figure 2).

It was first thought that the degree of aggregation of two vesicle membranes could be evaluated by knowing the interaction energy between the two vesicles. For this purpose, we have calculated the surface charge density σ (the fraction f of bound ion to phospholipid), the surface potential [Gouy–Chapman double-layer potential, $\Phi(0)$], and the interaction energy[2] $G(1/\kappa)$ for two vesicle membranes when the vesicles are separated by the Debye distance as a function of both monovalent and divalent cations and have examined how they change as the concentrations of both monovalent and divalent cations are varied. The formulas used to calculate these values are given in the Appendix. By comparing these calculated changes to the actual results obtained in this study, it should be possible to clarify the roles of these three parameters in cation-induced PS vesicle aggregation.

The results of these calculations, using Ca^{2+} as a representative example, are given in Table I.[3] The calculations indicate that the surface potential, $\Phi(0)$, of the PS membrane becomes less negative when the concentration of one cation (either monovalent or divalent) is increased, the other cation concentration being kept constant. On the other hand, although both the surface charge density and the interaction energy (as calculated from the electrostatic repulsive interaction energy, since it is a divalent ion) likewise decrease when the divalent cation concentration is increased at a constant monovalent cation concentration, they both increase when the monovalent cation concentration is increased at a constant divalent cation concentration. If a decrease in the surface charge density or interaction energy was governing the aggregation process, one should experimentally find that a higher threshold concentration of divalent cations was needed to induce PS vesicle aggregation as the monovalent cation concentration was increased. As shown in Figures 3 and 4, however, the opposite was actually observed.

From the experimental data and theoretical results, the surface potential seems to be well correlated with the degree of vesicle aggregation when the vesicle suspension solutions have similar ionic strength. However, the calculated surface potential values, which correspond to the threshold concentrations for aggregation, are very different for divalent cation-induced (−57 mV), monovalent cation-induced (−36 mV), and pH-induced (−62 mV) aggregation cases. In addition, the differences in the degree of divalent cation induced aggregation observed in the presence of Na^+ or TMA^+ (Figure 4) cannot be sufficiently explained in terms of surface potential.

Together these differences between the theoretical and experimental results suggest that PS vesicle aggregation cannot be satisfactorily explained simply in terms of static properties such as surface potential, surface charge density, or interaction

[2] As stated in the Appendix, the total interaction energy for two lipid vesicles separated by distance d can be expressed as the sum of the attractive interaction energy due to the van der Waals energy and the electrostatic repulsive energy due to double-layer interactions [$G(d) = G^{el}(d) + G^{A}(d)$; eq 8]. For divalent cations in the millimolar concentration range, the attractive energy, eq 7, was found to be almost constant for all cases of vesicle aggregation in this study. The way in which the total interaction energy changes therefore is determined by the electrostatic repulsive energy, eq 6. For monovalent cation induced vesicle aggregation, however, the attractive energy was not constant, and both terms had to be considered. It should also be noted that although the formula used for this calculation contains a number of approximations in its derivation for the electrostatic repulsive energy and therefore may not give definitive energy values, it should nevertheless indicate the manner in which this value changes. Since the equation expressing the interaction energy at short distances is not accurate where the highest value of the interaction energy would occur, we have estimated the energy at the Debye distance, in which the equation is more accurate. However, the choice of such a separation distance to estimate the interaction energy is rather arbitrary.

[3] Tables for all of the divalent cations, with both Na^+ and TMA^+ as the monovalent cations, as well as tables for monovalent cations only, and pH effects are available on request.

Table I: Electrostatic Quantities of a Phosphatidylserine Membrane as a Function of Divalent Ion Concentrations[a]

(A) Phosphatidylserine Suspension in Several NaCl Concentrations with Variation of Ca^{2+} Concentrations[b]

	NaCl								
	100 mM			10 mM			1 mM		
Ca^{2+} (mM)	$\Phi(0)$ (mV)	f	$G'(1/\kappa)$[c]	$\Phi(0)$ (mV)	f	$G'(1/\kappa)$	$\Phi(0)$ (mV)	f	$G'(1/\kappa)$
0.01	−81.6	0.367	0.0618	−114.3	0.239	0.026 2	−124.8	0.140	0.008 97
0.1	−73.9	0.314	0.0451	−92.3	0.173	0.013 6	−97.5	0.126	0.007 25
0.5	−63.0	0.251	0.0289	−74.9	0.145	0.009 6	−77.8	0.122	0.006 78
1.0	−57.4	0.224	0.0230	−67.1	0.137	0.008 61	−69.2	0.120	0.006 65
2.0	−51.4	0.199	0.0182	−59.2	0.131	0.007 84	−60.6	0.119	0.006 53
3.0	−47.7	0.186	0.0158	−54.4	0.128	0.007 48	−55.6	0.118	0.006 45
4.0	−45.0	0.177	0.0143	−51.0	0.126	0.007 25	−52.0	0.118	0.006 39
5.0	−42.7	0.170	0.0132	−48.4	0.124	0.007 07	−49.3	0.117	0.006 33
6.0	−41.2	0.165	0.0124	−46.3	0.123	0.006 94	−47.0	0.117	0.006 28
7.0	−39.7	0.161	0.0118	−44.4	0.122	0.006 82	−45.2	0.117	0.006 23

(B) Phosphatidylserine Suspension in Several TMA^+ Concentrations with Variation of Ca^{2+} Concentrations[d]

	TMA^+								
	100 mM			10 mM			1 mM		
Ca^{2+} (mM)	$\Phi(0)$ (mV)	f	$G'(1/\kappa)$	$\Phi(0)$ (mV)	f	$G'(1/\kappa)$	$\Phi(0)$ (mV)	f	$G'(1/\kappa)$
0.01	−95.4	0.493	0.1219	−115.8	0.247	0.028 2	−124.9	0.140	0.009 03
0.1	−79.1	0.352	0.0584	−97.6	0.174	0.013 9	−97.6	0.126	0.007 26
0.5	−65.1	0.264	0.0324	−75.1	0.146	0.009 74	−77.8	0.122	0.006 79
1.0	−58.8	0.232	0.0249	−67.3	0.137	0.008 67	−69.2	0.120	0.006 66
2.0	−52.3	0.204	0.0192	−59.2	0.131	0.007 88	−60.6	0.119	0.006 53
3.0	−48.4	0.189	0.0165	−54.5	0.128	0.007 51	−55.6	0.119	0.006 45
4.0	−45.6	0.180	0.0149	−51.1	0.126	0.007 27	−52.0	0.118	0.006 39
5.0	−43.4	0.173	0.0137	−48.5	0.129	0.007 09	−49.3	0.118	0.006 33
6.0	−41.7	0.167	0.0129	−46.3	0.123	0.006 95	−47.0	0.117	0.006 28
7.0	−40.2	0.163	0.0122	−44.5	0.122	0.006 84	−45.1	0.117	0.006 23

[a] Calculated values of the surface potential $\Phi(0)$, the fraction of surface charge density, $f = \sigma/\sigma^{int}$, and the electrostatic interaction energy, G', of the two phosphatidylserine vesicles separated by the Debye distance ($=1/\kappa$), where the assumed surface charge density of the membrane, σ^{int}, is $-e/65$ Å^2, the outside radius of the vesicle is 150 Å, and the dielectric constant of a vesicle suspension, ϵ, was taken as 80. [b] Binding constants of Ca^{2+} and Na^+ used in the calculation were 30 and 0.6 M^{-1}, respectively. [c] $G' = G^{el}/(2.55 \times 10^{-12}$ erg). [d] Binding constants for Ca^{2+} and TMA^+ were 30 and 0.05 M^{-1} (Ohki & Kurland, 1981), respectively. The assumed pH of the above vesicle suspension solutions was 7.0. $G^{el} = (2.55 \times 10^{-12}$ erg$)G'$.

energy at a particular distance between vesicles. Instead, a theoretical framework that takes dynamic interactions into account may be necessary.

Such dynamic interactions have been discussed by Verwey & Overbeek (1948), who suggested that in order for two particles to adhere to one another, it may be necessary that the driving force acting on a particle be equal to or greater than the repulsive force corresponding to the steepest part of the repulsive interaction energy curve. Since it is not possible to obtain an accurate formulation for the repulsive and attractive interaction energies for the very small membrane separation distances where the steepest part of the repulsive interaction curve may occur, we evaluated the repulsive force (slope of interaction energy) at the Debye distance for two interacting membranes in an electrolyte solution at its threshold concentration for vesicle aggregation. Such a repulsive force, $F(1/\kappa)$, is obtained from eq 8 (see Appendix).

$$F(1/\kappa) = -\left.\frac{\partial G(d)}{\partial d}\right|_{d=1/\kappa} = \left(\frac{8\pi^2 r\sigma^2}{\epsilon\kappa}\,\frac{1}{e-1}\right) - \left.\frac{\partial G^A}{\partial d}\right|_{d=1/\kappa}$$

where σ is the surface charge density of the membrane and κ is the Debye constant $[\kappa = [[4\pi e^2 N/(\epsilon kT)]\Sigma_j(Z_j^2 C_j)]^{1/2}]$ at the ionic environment corresponding to the threshold concentration for vesicle aggregation.

As an example, for the Ca^{2+}-induced aggregation condition, $Ca^{2+}_{threshold}$ = 1 mM Ca^{2+} and 100 mM NaCl, $F(1/\kappa)$ was calculated to be about 3.82×10^{-5} dyn by using the previously determined binding constants of 30 M^{-1} for Ca^{2+} and 0.6 M^{-1} for Na^+ to PS membranes (Ohki & Kurland, 1981), a dielectric constant ϵ of 80, and assuming an outer and inner radii of vesicles of 150 and 100 Å, respectively.

To test the hypothesis that it is the slope of the interaction energy (repulsive force) that is involved in governing PS vesicle aggregation, we can work backward, assuming that the slopes of the interaction energy at the Debye distance are the same at the threshold concentrations for the various cations, and calculate what the cation binding constants would be. These calculated values could then be compared to those obtained by other methods. With the value for $F(1/\kappa)$ of 3.82×10^{-5} dyn obtained from the Ca^{2+} case mentioned above, the binding constants for the other divalent cations were calculated to be 49 M^{-1} for Mn^{2+}, 37 M^{-1} for Ba^{2+}, 25 M^{-1} for Sr^{2+}, and 10 M^{-1} for Mg^{2+}. These values compare favorably with those found with other methods, 50 M^{-1} for Mn^{2+} (S. Ohki, unpublished results), 10 M^{-1} for Mg^{2+} (Ohki & Kurland, 1981), and 4 M^{-1} for Mg^{2+} (Nir et al., 1978).

Equivalent calculations were made for the monovalent cations by assuming that the slope of the repulsive interaction energy obtained at the Debye distance for Ca^{2+} was also the same for monovalent cations, and by using monovalent threshold concentrations of 0.8 M for Na^+, 1.1 M for Li^+, and 1.5 M for K^+. The monovalent cation binding constants then calculated were 0.6 M^{-1} for Na^+, 0.4 M^{-1} for Li^+, and 0.2 M^{-1} for K^+. Again, these values compare favorably with those obtained for PS membranes by other methods: (1) 0.6 M^{-1}

for Na^+ obtained from surface potential measurements (Ohki & Kurland, 1981); (2) 0.8 M^{-1} for Na^+ determined from equilibrium dialysis experiments (Nir et al., 1978); (3) 1.2–0.4 M^{-1} for Na^+ from ^{23}Na NMR studies (Kurland et al., 1979); (4) 0.6 M^{-1} for Na^+, no binding for TMA^+, and 0.15 M^{-1} for K^+ derived from electrophoretic mobility measurements of multilamellar PS liposomes (Eisenberg et al., 1979). On the other hand, the association constant for Li^+ was found to be less than that for Na^+ in this study, which is reversed from that reported by Hauser et al. (1970) and Eisenberg et al. (1979).

Finally, the association constant of H^+ to PS membranes was calculated, again by using the same $F(1/\kappa)$ value (3.82×10^{-5} dyn) obtained for Ca^+, a Na^+ binding constant of 0.6 M^{-1}, and a threshold pH value of 2.6 for H^+-induced PS vesicle aggregation in the presence of 0.1 M NaCl. The value obtained for the H^+ association constant was 100 M^{-1}.

The good agreement observed between the binding constants obtained in this study from the repulsive force equation, $F(1/\kappa)$, and those reported in other studies using various methods strongly supports the hypothesis that PS vesicle aggregation is related to the slope of the interaction energy (repulsive force) at the Debye distance for two interacting membranes. The only obvious inconsistency found was the reversal in the order of the Li^+ and Na^+ binding constants, which reflects the order of their threshold concentrations for vesicle aggregation. This reversal was not due to differences in the kinetics of vesicle aggregation between these two ions but corresponded to a difference in cation selectivity. Such a difference might be due to the large concentration of monovalent ions used in this study (0.8 M) in contrast to that used in the Eisenberg et al. (1979) study or the fact that unilamellar vesicles were used in these experiments while multilamellar vesicles were employed by Eisenberg et al. (1979). A more likely possibility, however, is that the difference is the result of the relationship between the hydration properties of the cations and their interaction sites as described by Eisenman (1962). This possibility is presently being investigated. It should be noted that the above discussion is applicable to acidic phospholipid membrane systems but probably not to other lipid membranes (e.g., phosphatidylcholine) whose surfaces possess a greater degree of structural water associated with the lipid head groups (Hauser, 1975; LeNeveu et al., 1976).

In summary, it seems unlikely, on the basis of our experiments and calculations, that PS vesicle aggregation can be satisfactorily explained by such electrostatic quantities as surface potential, surface chage density, or the total interaction energy at a particular distance of vesicle membrane separation. Instead, a theoretical treatment of PS vesicle aggregation that is based upon the dynamic interactions of the vesicle membranes seems to be necessary. Such dynamic interactions are included in the formulation of the repulsive force, $F(1/\kappa)$, acting between two membranes separated by the Debye distance proposed in this study. This formulation, as determined by the results obtained, is much better than the three electrostatic parameters discussed above in explaining cation-induced aggregation of PS vesicles. In addition, it is possible, with this formulation, to determine the binding constants of many, if not all, cations to PS membranes.

Finally, it should be mentioned that although vesicle aggregation is a necessary step for membrane fusion, the biophysical parameters responsible for membrane fusion may be both different and distinct from those for membrane aggregation. Experiments to provide such information about the mechanism of membrane fusion are presently being performed in our laboratory.

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Appendix

The Gouy–Chapman double-layer potential (which we term here "surface potential") at the membrane–electrolyte interface is expressed by

$$\sigma = \frac{1}{271}\left[\sum_i C_i\left(\exp\left[-\frac{ez_i\Phi(0)}{kT}\right] - 1\right)\right]^{1/2} \quad (1)$$

where σ is the surface charge density (charge per Å^2), C_i the molar concentration of the ith ionic species in the bulk phase, z_i the valency of the ith ion, $\Phi(0)$ the surface potential at the membrane interface, k the Boltzmann constant, and T the temperature, 24 °C.

We assume that one divalent ion would bind with two phospholipids simultaneously and one monovalent ion with one phospholipid independently. Then, we have the following reactions:

$$C^+ + A^- \overset{K_1}{\rightleftharpoons} C^+A^- \qquad C^{2+} + A^{--} \overset{K_2}{\rightleftharpoons} C^{2+}A^{--} \quad (2)$$

where A^{--} is two phospholipid groups and K_1 and K_2 are the association constants for the above reactions.

$$K_1 = \frac{[C^+A^-]}{[C^+][A^-]} \qquad K_2 = \frac{[C^{2+}A^{--}]}{[C^{2+}][A^{--}]} \quad (3)$$

Here, $[A^{--}] = [A^-]/2$, when $[A^-]$ is the surface concentration of phospholipid molecules.

Then, the surface charge density σ is expressed by

$$\sigma = \frac{\sigma^{int}}{1 + K_1[C^+]\exp\left(\frac{-e\Phi(0)}{kT}\right) + K_2[C^{2+}]\exp\left(\frac{-2e\Phi(0)}{kT}\right)} \quad (4)$$

where σ^{int} is the surface charge density with no ion binding.

Using eq 1 and 4 and knowing all ionic concentrations in the bulk phase and the initial surface charge density of the phosphatidylserine bilayer ($-e/65$ Å^2), the surface potential is calculated by a computer (CDC 4000 at SUNY/Buffalo), giving K_1, the association constant for monovalent ions, and K_2, the association constant for divalent ions.

The fraction of the surface charge density, f, is defined by

$$f = \sigma/\sigma^{int} \quad (5)$$

By using the calculated surface charge densities, the free energy due to the electrostatic repulsive force acting between two lipid vesicles separated at a distance d is calculated with the equation (Wiese & Healy, 1970)

$$G^{el}(d) = \frac{8\pi^2 r}{\epsilon\kappa^2}\sigma^2 \ln\left[\frac{1}{1 - \exp(-\kappa d)}\right] \quad (6)$$

where κ is $[[4\pi e^2 N/(\epsilon kT)]\sum_j (Z_j^2 C_j)]^{1/2}$.

The van der Waals interaction energy of two spherical cells (the outer radius is r and the thickness is h) at separation d is expressed (Ohsawa, 1981) as

$$G^A(r,h,d) = V(r,r) + V(r-h, r-h) - 2V(r-h, r) \quad (7)$$

where

$$V(r_1,r_2,d) = (-A/6)\left[\frac{2r_1r_2}{(d + r_1 + r_2)^2 - (r_1 + r_2)^2} + \frac{2r_1r_2}{(d + r_1 + r_2)^2} + \ln\frac{(d + r_1 + r_2)^2 - (r_1 + r_2)^2}{(d + r_1 + r_2)^2}\right]$$

where A is the Hamaker's constant and r_1 and r_2 are the radii of two spherical cells, respectively.

Therefore, the total interaction energy for two opposing membranes is expressed as

$$G(d) = G^{el}(d) + G^{A}(d) \quad (8)$$

In the case of two monovalent cations present in the suspension solution, the binding reaction equations are similar to the above, and the surface charge density is expressed by

$$\sigma = \sigma^{int}/\left[1 + K_1'[C_1^{+\prime}]\exp\left(-\frac{e\Phi(0)}{kT}\right) + K_1''[C_1^{+\prime\prime}]\exp\left(-\frac{e\Phi(0)}{kT}\right)\right]$$

instead of eq 4, where K_1' and K_1'' are the binding constants for the two species of monovalent cation.

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