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Evidence for a Role of Phosphatidyl Ethanolamine as a Modulator of Membrane-Membrane Contact

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Summary. Phosphatidyl ethanolamine (PE) is shown to be effective in producing membrane aggregation. The aggregation of PE and PE/PC (phosphatidyl choline) mixed vesicles was studied as a function of pH and cation composition of the medium. The kinetics and equilibria were studied in stopped-flow rapid mixing experiments, in which PE vesicles prepared at pH 9.2 were "jumped" to pH 7.

H^+ ions protonate PE^- and promote vesicle aggregation in a cooperative fashion. Vesicles containing PC have a decreased tendency to aggregate compared to pure PE vesicles. The apparent rate constant for aggregation was about two orders of magnitude below that for diffusion controlled aggregation and was virtually the same for PE and PE/PC mixed vesicles.

A theoretical description of equilibrium for vesicle aggregation is developed in terms of three parameters: the equilibrium constant for the protonation of PE (K_A), the equilibrium constant for aggregation (K_{eq}) and the number of PE molecules in an effective area that the two vesicles must interact in order to aggregate (N_{eff}). These parameters are compared with values and trends expected for electrostatic calculations based on dipolar repulsion and short-range binding, to which hydrogen bonding may contribute. The results are interpreted in a self-consistent fashion to indicate: (i) that PE and PC mix randomly, (ii) that head-to-tail binding occurs between PE(PC) molecules on apposing vesicles, (iii) that electrostatic screening accounts for the decrease in K_A as a function of the molar fraction of PC per vesicle, (iv) that the PE must be 90 % protonated before aggregation can occur, and (v) that for all the lipid systems we considered, the point at which the extent of dimerization is half maximal is close to the physiological pH, indicating that PE may have a regulatory effect in the aggregation of biological systems.

The question of what role lipids and lipid bilayer regions play in the aggregation and fusion of biomembranes remains an important question in membrane biology. It has been speculated that lipids may play a primary role in exocytotic fusion reactions of storage vesicles and the plasma membrane (*cf.* Llinas & Heuser, 1977). A primary role would involve direct contact of the lipid bilayer portions of the membrane, and some of the mechanistic features of the reaction (pH, cation, temperature dependence, rate, etc.) should also be observed with phospholipid vesicles

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derived from these biomembranes. If the lipids play only a secondary role in the aggregation and fusion reactions, such as modulating the strength of interaction through long-range interactions (van der Waals attraction, electrostatic and dipolar repulsion, etc.), then fewer similarities should be expected. Clearly, a detailed knowledge of the short and long-range interactions of lipids is required if firm conclusions are to be made.

The aggregation and fusion reactions of phosphatidyl choline (PC) and of the acidic phospholipids (phosphatidic acid, PA; phosphatidyl serine, PS) have been well studied. Vesicles from PC, a major component of all biomembranes, do not engage in aggregation or fusion reactions under normal conditions. The interactions of PC polar head groups are "repulsive", and large energies must be expended to bring PC bilayers up to within a few Å of each other (LeNeveu, Rand & Parsegian, 1976; LeNeveu *et al.*, 1977). Membranes composed of PA^- or PS^- do not aggregate at physiological monovalent electrolyte concentrations due to the electrostatic repulsion of their charged surfaces (Lansman & Haynes, 1975). Addition of millimolar concentrations of divalent cations brings about aggregation on the time scale of seconds (Lansman & Haynes, 1975) and fusion on the time scale of tens of minutes (Papahadjopoulos *et al.*, 1974, 1976). Incorporation of PC into PA^- or PS^- drastically reduces the rate and extent of these reactions, (Lansman & Haynes, 1975; Papahadjopoulos *et al.*, 1974, 1976) such that aggregation reactions of secretion granules cannot be accounted for by acidic phospholipids and PC in the ratio of mole fractions found in these membranes (Morris, Chiu & Haynes, 1979). However, phospholipid vesicles derived from chromaffin (secretion) granule membranes did show a cation-induced aggregation reaction, and this was shown to be due to the influence of the high mole fraction of phosphatidyl ethanolamine (PE) in these membranes (Morris *et al.*, 1979).

In the present study, we show that PE induces a strong aggregation tendency in model membranes and that the energetic basis of this is a transmembrane head-to-tail bonding of the polar head groups. This is similar to the intramembrane hydrogen bonding interaction of PE for which we have produced evidence (Haynes & Staerk, 1974). In the present study, we describe the equilibrium and kinetics of aggregation of PE and PE/PC vesicles. The system is characterized in terms of the PE^- protonation equilibrium, the minimal effective interaction area for vesicle aggregation, and in terms of the equilibrium constant for aggregation and the forward and reverse rates. The energetic balance between "re-

pulsive" interactions of PC and "attractive" interactions of PE is described. The evidence presented will show that PE, in the concentrations in which it is found, can be an effective modulator of membrane aggregation.

Materials and Methods

The phospholipids were purchased from Koch-Light Laboratories, dipalmitol-L-3-phosphatidyl ethanolamine (DPPE) No. 49731; from Sigma Laboratories, dimyristol-L-α-phosphatidyl choline No. P-5141, commercial grade (from bovine brain), L-α-phosphatidyl ethanolamine No. P-4264; from Fluka Laboratories, dimyristol-L-α-phosphatidyl ethanolamine (DMPE) No. 629L17; and from Calbiochem Laboratories, dimyristol-L-α-phosphatidyl ethanolamine (DMPE) No. 524634. The vesicles were prepared by sonicating 6 mg of lipid for approximately 2 min in 2 ml of 10 mM Tris buffer, pH 9.5. Sonication of the vesicles for 10 min produced no appreciable differences in the results. The vesicles were sonicated using a Heat Systems W185D Sonifier at stage 5 (nominal power output 60 W), and kept in a heated bath (65 °C) above their phase transition temperatures (DMPE $T_c = 49$ °C, DPPE $T_c = 62$ °C; Blume & Ackermann, 1974).

Rapid mixing experiments were performed with an Aminco-Morrow Stopped Flow Apparatus (Cat. No. 4-8409) in the transmittance mode at 56 °C. The samples were allowed to come to thermal equilibrium with the stopped-flow apparatus. The pH of all final mixed solutions was temperature corrected. The progress curve for the change in scattering for 0.06 mg/ml vesicles could be expressed as the sum of two processess: the first process being the only one studied quantitatively and having a $t_{\frac{1}{2}}$ of approximately 2 sec. The beginning of the second process interfered with the evaluation of the first at times greater than four $t_{\frac{1}{2}}$. The end point was determined at the start of the second process and the extent of the first reaction was calculated after extrapolating the second process to zero time and subtracting it off. The extent of the first reaction, α, was calculated according to the expression $\alpha = \log(I_R/I_P)/\log(I_O/I_R)$, where I_R is the transmitted intensity for the reactants, I_P is the transmitted intensity for the products (after subtraction of the higher order processes) and I_O is the intensity between light and dark, normalized to 10 V.

The steady-state Ca^{2+} and K^+ results were obtained from scattering at 330 nm using a Hitachi-Perkin Elmer MPF3 spectrophotometer. The solution preparations were the same as in the rapid mixing experiments. All curve fitting was done using the PROPHET computer system.

Results

PE vesicles aggregate avidly at pH 7. When PE vesicles buffered at pH 9.2 are rapidly mixed with a solution of HCl (0.01 M) to give a final pH of 6.8 a change in the optical transmittance occurs. In Fig. 1 a typical time course is displayed, this reaction can be reversed by changing the pH to 9.2 (e.g., *see* Fig. 2). As in previous work (Lansman & Haynes, 1975), these results are taken to indicate the light-scattering change is a

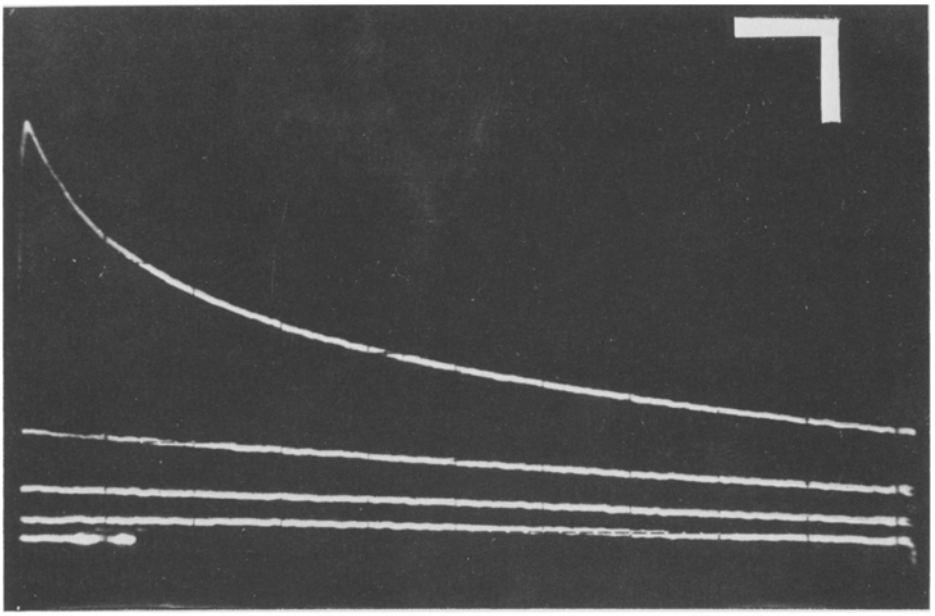

Fig. 1. A stopped-flow progress curve showing the decrease in transmittance at 330 nm due to vesicle aggregation is presented. The ordinate is transmitted intensity given in units of voltage (0.5 V/div), and the abscissa is time after mixing (2 sec/div). Syringe *A* contained DPPE vesicles (0.12 mg/ml) in 10 mM Tris, pH 9.0; syringe *B* contained HCl:water mixture so that upon mixing with *A* the final pH was lowered to 6.3. The oscilloscope was set in the repetitive sweep mode

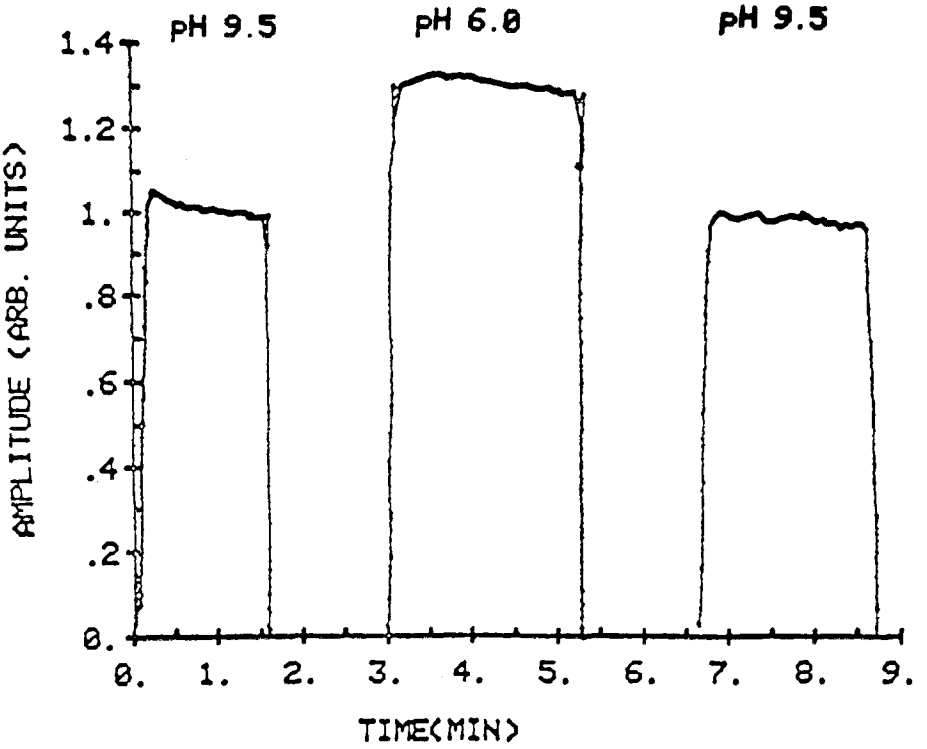

Fig. 2. The reversibility of DPPE vesicles is indicated in a recorder trace of the 90° light scattering amplitude *vs.* time at different pH values. The lipid concentration is 0.1 mg/ml in 0.01 M Tris buffer. The temperature of the system is 56 °C

result of an aggregation reaction. The dominant process is the dimerization of two vesicles which follows a time course given by (Lansman & Haynes, 1975)

$$\frac{\alpha}{1-\alpha} = \frac{2[PL]\,k_{app}}{N_{PL}} \cdot t, \tag{1}$$

where α is the degree of advancement of the reaction, $\alpha = 2[V_2]/[V_0]$, t is the reaction time, $[PL]$ is the molar concentration of phospholipid, N_{PL} is the number of phospholipid molecules per vesicle, and k_{app} is the apparent "bimolecular" rate constant for vesicle dimerization defined by the stoichiometric equation

$$V + V \underset{k_{-app}}{\overset{k_{app}}{\rightleftharpoons}} V_2, \tag{2}$$

Figure 3 shows that Eq. (1) is obeyed for $0 \leqq \alpha \leqq 0.5$.

Aggregation as a Function of pH

In Figs. 4 and 5 the equilibrium value of degree of advancement of the dimerization reaction, α, is plotted as a function of pH, for dipalmitol-

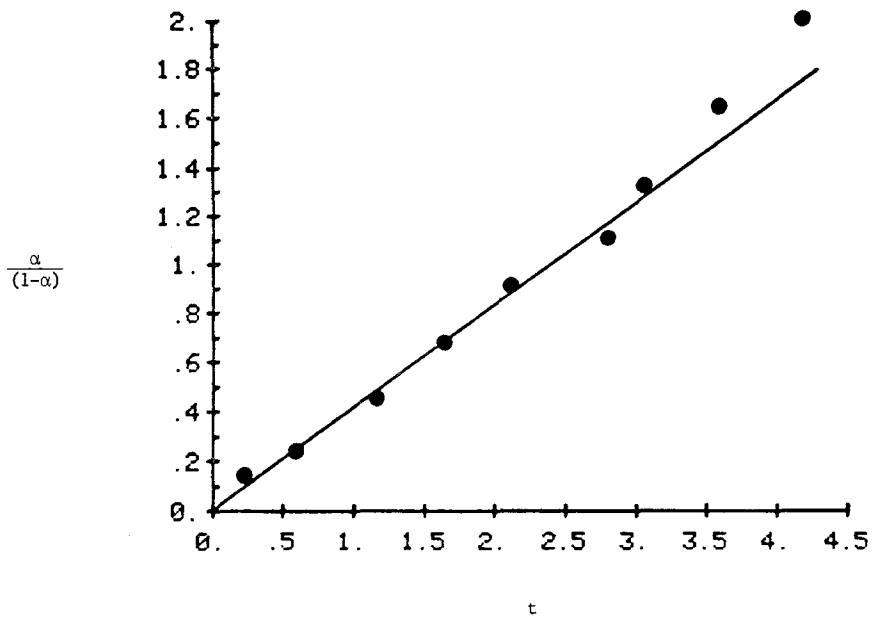

Fig. 3. A second-order plot of the progress curve given in Fig. 1. The solid line represents the straight-line fit according to Eq. (1) of the text

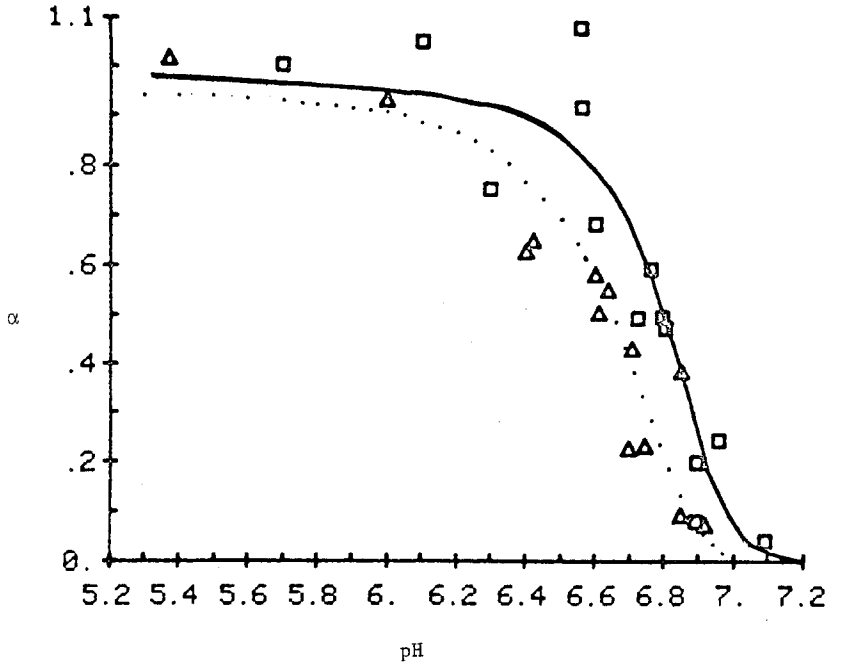

Fig. 4. The extent of dimerization, α is plotted against pH for DPPE. The squares (□) represent 100% PE and the triangles (△) represent mixed PC/PE vesicles (87% PE). The solid and dotted lines are the theoretical fit of Eq. (10) to the squares and triangles, respectively

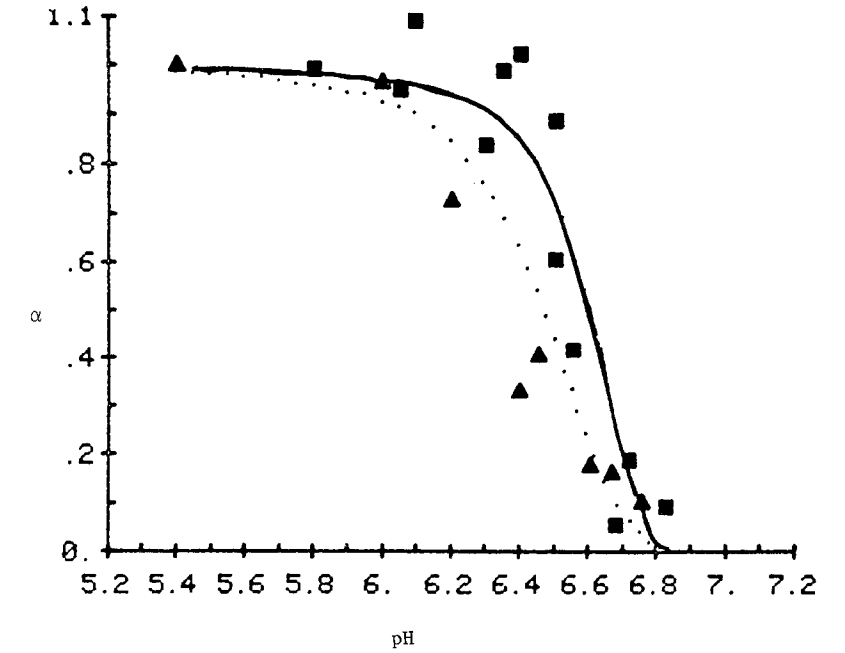

Fig. 5. The extent of dimerization, α is plotted against pH for DMPE. The squares (■) represent 100% PE and the triangles (▲) represent mixed PC/PE vesicles (80% PE). The solid and dotted lines are the theoretical fit of Eq. (10) to the squares and triangles, respectively

phosphatidyl ethanolamine (DPPE) and dimyristol-phosphatidyl ethanolamine (DMPE) at 56 °C, respectively. The solid curves are theoretical fits to the data and are explained in the discussion section. The prominent features of each figure are that an increase in H^+ concentration promotes vesicle aggregation in a cooperative fashion, and that vesicles containing PC have a decreased tendency to aggregate when compared to pure PE vesicles.

In Figs. 4 and 5 we see that the aggregation curves for pure DMPE are shifted to a lower pH compared to DPPE. To investigate whether this effect is due to differences in the lipid phase transition temperature, T_c (DMPE, $T_c = 49$ °C; DPPE, $T_c = 62$ °C; Blume & Ackermann, 1974), we ran a number of experiments on pure DMPE at 30 °C. We found that at 30 °C the DMPE curve shifts to higher pH by about 0.45 pH units.

Similar titration experiments were run on commercial grade PE and, in fact, aggregation occurs but is shifted to much lower pH values (*ca.* pH 5). For vesicles containing a molar fraction of PC greater than 0.4, aggregation could not be induced.

Calculation of k_{app}

In Fig. 6 we plot $1/t_{\frac{1}{2}}$ *vs.* initial vesicle concentration ($\alpha(t_{\frac{1}{2}}) = 0.5$). A good fit to the data is obtained by using Eq. (1). By knowing the slope of the curves of Fig. 6 we can calculate k_{app} from Eq. (1); k_{app} is *ca.* $2.7 \times 10^7\,\text{M}^{-1}\text{sec}^{-1}$ for DPPE and *ca.* $2.0 \times 10^7\,\text{M}^{-1}\,\text{sec}^{-1}$ for DMPE, where N_{PL} is *ca.* 10^4 lipids/vesicle (*see* discussion section on N_{eff}). The value of k_{app} is relatively independent of the PC/PE ratio.

The value of k_{app} is the same order of magnitude as that obtained in Ca^{2+} aggregation of PA vesicles (Lansman & Haynes, 1975). The difference in k_{app} from a diffusion controlled value, k_{diff} ($= 8\pi NRD$) (Lansman & Haynes, 1975) of $6.8 \times 10^9\,\text{M}^{-1}\,\text{sec}^{-1}$ indicates that in order for vesicles to aggregate they must overcome an energy barrier.

Cation-Induced Aggregation

Experiments were performed using 1 mM Ca^{2+} and 10 mM K^+ at both high (9) and low (6.3) pH. Table 1 is a compilation of the experimental $t_{\frac{1}{2}}$ values. From this table one sees that Ca^{2+} induced aggregation at the high pH was more rapid than at the lower pH. At the final equilibrium

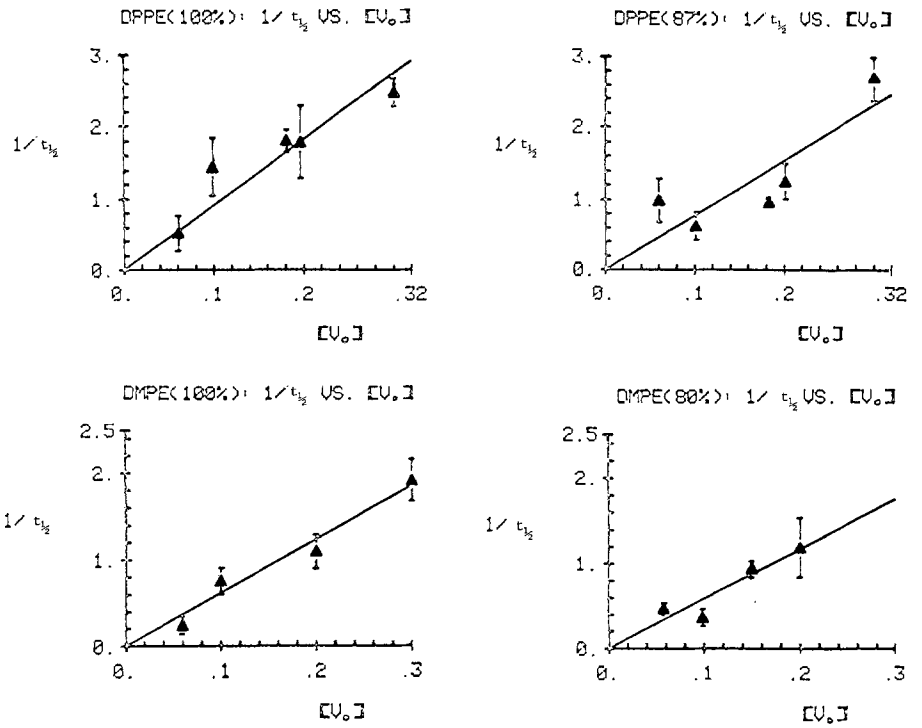

Fig. 6. The dependence of $1/t_{\frac{1}{2}}$ on phospholipid vesicle concentration. The experimental conditions were identical to those of Fig. 1

Table 1. The effect of cation on $t_{\frac{1}{2}}$ of DPPE[1]

pH	Cation	$t_{\frac{1}{2}}$ (sec)
9.0	1 mM $CaCl_2$	0.78 ±0.1
9.0	10 mM KCl	None
9.0	–	None
6.3	1 mM $CaCl_2$	1.4 ±0.1
6.3	10 mM KCl	1.88 ±0.2
6.3	–	1.2 ±0.2

[1] α has been taken equal to 1 and $T = 56$ °C.

the amplitude of aggregation of Ca^{2+} plus H^+ addition (pH 7) is greater than the efffect of Ca^{2+} alone (pH 9). Presumably Ca^{2+} and H^+ competition occurs at the site of the deprotonated amine, and the binding affinity of the H^+ is significantly greater than that of Ca^{2+}. The aggregation effects were reversible when NaOH and/or EDTA were added at any time during the 4-min run.

The addition of 10 mM K^+ to PE vesicles does not induce aggregation at high pH. At low pH, K^+ seems to slightly screen the effect of the H^+ ions and slow down the aggregation reaction (*cf.* Table 1).

Discussion

The data of Figs. 4 and 5 indicate that the equilibrium constant for the aggregation process is a function of pH. As the pH is lowered towards physiological pH (*ca.* 7.0), the amine residue of the PE is protonated. We postulate that aggregation results from lowering the net charge per vesicle and thereby reducing intervesicular repulsion. This dependence of dimerization on the hydrogen concentration will be considered in the following section.

Description of Equilibrium for the Vesicle Aggregation

The protonation of the PE is fast compared to aggregation. This is indicated by the fact that at high $[H^+]$ the $t_{\frac{1}{2}}$ for aggregation is constant, showing the protonation of the amine group is not rate limiting. The stoichiometric equation for protonation is

$$PE^- + H^+ \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} PE \tag{3}$$

where PE^- represents a deprotonated PE, and k_0 is the bimolecular rate constant. An equilibrium association constant[1], K_A, can be defined

$$K_A = k_0/k_{-0} = [PE]/([PE^-][H^+]). \tag{4}$$

It follows from Eqs. (3) and (4) that the probability of PE being in the protonated form is given by

$$[PE]/[PE]_{total} = K_A[H^+]/(1 + K_A[H^+]) \tag{5}$$

where $[PE]_{total} = [PE] + [PE^-]$.

During the aggregation process the forces on a vesicle are predominantly determined from the lipid in a minimal interaction area which lies in apposition to its partner vesicle. We assume that a number,

[1] K_A will be a function of the surface charge density and is related to the intrinsic K_A ($K_{A,I}$) by the Gouy-Chapman theory as described in the text discussion of K_A.

N_{eff}, of PE molecules must be protonated in an interaction area so that the vesicles are capable of aggregation. Therefore, the probability that a vesicle is capable of aggregating is given by

$$\underline{P} = ([\text{PE}]/[\text{PE}]_{\text{total}})^{N_{\text{eff}}}. \tag{6}$$

Here it is assumed that the effect of surrounding lipids have been incorporated in the association constant K_A. Therefore, the total probability $\underline{P}$ (Eq. (6)) is given by a product of independent terms. This approximation is identical to those made in early studies of allosteric transitions (Monod, Jeffries & Changeux, 1965) when describing ligand binding. One might expect that any higher order effects, due to particle-particle correlations and screening, could be incorporated as a re-normalized K_A, which at the very least can be considered an empirical fit constant.

In our system the pH-dependent equilibrium constant for aggregation $K_{\text{eq}}(\text{pH})$ is determined from the bimolecular rate constants k_{app} and $k_{-\text{app}}$ of the stoichiometric equation

$$V + V \underset{k_{-\text{app}}}{\overset{k_{\text{app}}}{\rightleftharpoons}} V_2 \tag{7}$$

where V_2 represents the dimerized vesicles. Since $k_{-\text{app}}$ is a measure of the time vesicles satisfying the protonation requirement are aggregated, we expect that it is independent of pH. However k_{app} is smaller than the bimolecular rate constant for fully protonated vesicles, k^f_{app}, by a factor $\underline{P}^2$. It follows that $K_{\text{eq}}(\text{pH})$ is given by

$$K_{\text{eq}}(\text{pH}) = \frac{k_{\text{app}}}{k_{-\text{app}}} = \underline{P}^2 \frac{k^f_{\text{app}}}{k_{-\text{app}}} = \underline{P}^2 K_{\text{eq}} \tag{8}$$

where K_{eq} is the equilibrium constant when $\underline{P} = 1$. From Eq. (7) $K_{\text{eq}}(\text{pH})$ is

$$K_{\text{eq}}(\text{pH}) = [V_2]/[V]^2. \tag{9}$$

Equating Eqs. (8) and (9), and employing the conservation of vesicles, $[V_0]$, we find

$$\frac{\alpha}{(1-\alpha)^2} = 2[V_0]\, K_{\text{eq}} \cdot \left(\frac{K_A[\text{H}^+]}{1 + K_A[\text{H}^+]}\right)^{2N_{\text{eff}}} \tag{10}$$

where $\alpha = 2[V_2]/[V_0]$, and $\underline{P}$ has been eliminated using Eqs. (5) and (6).

The solid and dotted lines of Figs. 4 and 5 are the theoretical fit of Eq. (10) to the data. The logarithm of Eq. (10) was fit, and an example of the fit is shown in Fig. 7 for 100% DPPE. The fit constants K_{eq}, K_A and N_{eff} are given in Table 2. This table shows that the constants are a function of PE mole fraction and that they decrease as the mole fraction of PC per vesicle increases. Below we interpret these results in terms of simple electrostatic arguments.

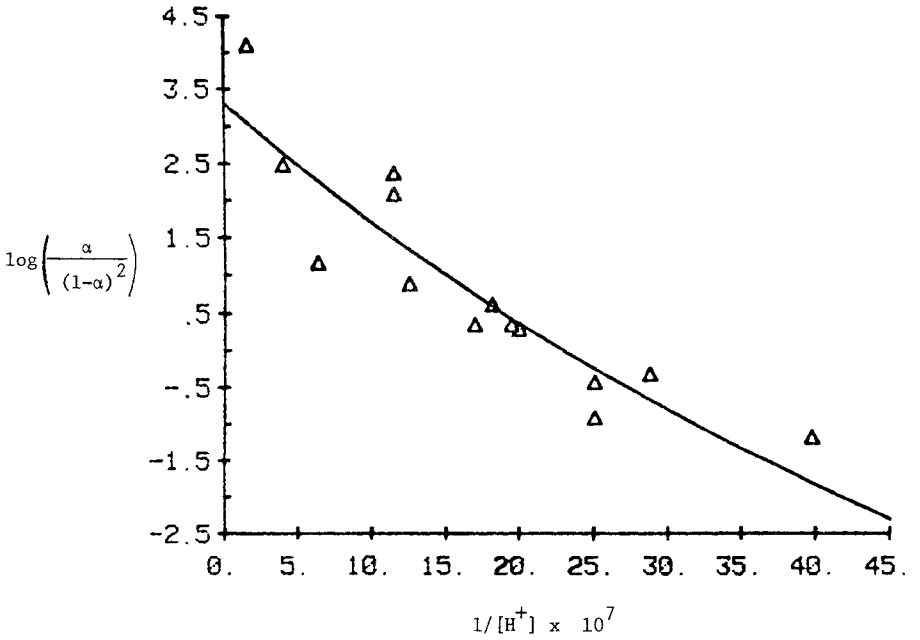

Fig. 7. The log $(\alpha/(1-\alpha)^2)$ is plotted against $1/[H^+] \times 10^7$. The triangles (△) represent the data for DPPE (100%) and the solid line represents the fit from Eq. (10) in the text

Table 2. Theoretical fit values to data

Lipid	%PE	K_{eq} (M^{-1})	K_A (M^{-1})	N_{eff}
DPPE	100	10^{11}	1.5×10^7	9.6
	87	2.5×10^{10}	9.49×10^6	7.0
DMPE	100	2.0×10^{12}	6.32×10^6	10.0
	80	6.3×10^{11}	1.4×10^6	3.8

Each K_{eq} was obtained by extrapolating the low pH values to infinite H^+ concentration. These values are accurate to within a factor of two. The parameters N_{eff} and K_A were then found by a least-squares fit of the data. N_{eff} is accurate to 10% and K_A to 20%.

The Equilibrium Constant for Vesicle Aggregation, K_{eq}

In this section we account for the absolute value of K_{eq}, and explain why it decreases as a function of mole fraction of PC. K_{eq} will be described by considering the kinetic rate constants k^f_{app} and k_{-app}.

As shown in the results section, k^f_{app} is smaller than the diffusion controlled rate constant by two orders of magnitude. This indicates (Lansman & Haynes, 1975; Morris *et al.*, 1979) the existence of an energy barrier, shown in Fig. 8 (e.g., Dean & Matthews, 1975), which a vesicle must overcome to form an aggregate. The rate constant for aggregation, k^f_{app}, will be reduced maximally (Verwey & Overbeck, 1948) by a factor of $\exp(E^*_t/kT)$, where E^*_t is the barrier height seen by the incoming vesicle, k is the Boltzmann constant, and T the absolute temperature. Similarly, the rate constant for disaggregation, k_{-app}, is reduced by

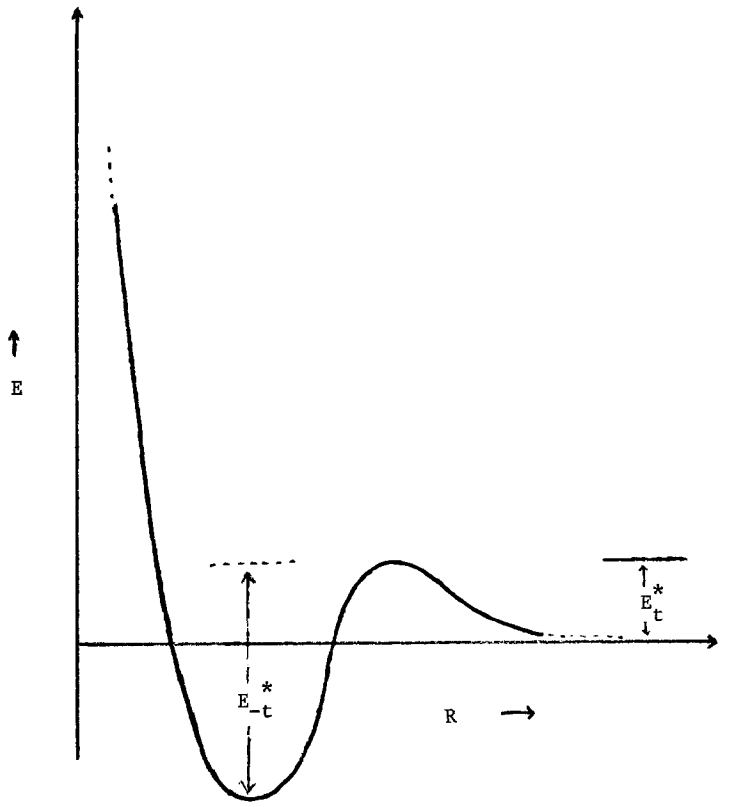

Fig. 8. The hypothetical potential E for vesicle aggregation is plotted against the distance between vesicles. E^*_t represents the maximal barrier height for an incoming vesicle, and E^*_{-t} represents the maximum barrier height for disaggregating vesicles

$\exp(E^*_{-t}/kT)$, where E^*_{-t} is the height seen by the disaggregating vesicle. Therefore, the existence of a potential barrier results in the rate constants given by

$$k_{app} = k_{diff} \exp(-E^*_t/kT) \tag{11}$$

and

$$k_{-app} = k_{-diff} \exp(-E^*_{-t}/kT) \tag{12}$$

where $k_{diff} \approx 6.8 \times 10^{+9}$ M^{-1} sec^{-1} and $k_{-diff} \approx 10^{+5}$ sec^{-1} (Smoluchowski, 1916; *cf.* Lansman & Haynes, 1975).

From the value of K_{eq} and k_{app} the potential energy E^*_t is found to be *ca.* 4.61 kT (3.01 kcal/mole) for both DPPE and DMPE, and E^*_{-t} is 20.72 kT (13.54 kcal/mole) for DPPE and 23.03 kT (15.05 kcal/mole) for DMPE. If one assumes that the repulsive barrier is electrostatic in nature then the ratio E^*_t/E^*_{-t} can be explained in terms of a dipole model. We assume that the PE heads are represented by macroscopic dipoles, and when two vesicles approach each other the dominant intervesicle interaction is from the dipoles that are directly apposed from each other. For separation distances greater than 14 Å, Colbow & Jones (1974) have demonstrated that the free energy of interaction between two dipole layers is proportional to the number of dipoles per unit area. Within this framework we assume the energy barrier is the sum of dipole-dipole interactions. Furthermore, we assume that if the correlation energy between planar dipoles is less than the dipole-dipole correlations between vesicles (for distances less than 14 Å) then the dipole approximation will hold. Therefore, the ratio E^*_t/E^*_{-t} is approximated by the barrier heights due to two approaching macroscopic dipoles. For the case of two dipoles, shown in Fig. 9, with a tilt angle of 45° (NMR and X-ray studies (Yeagle *et al.*, 1976; Hitchcock *et al.*, 1974) indicate that the PE polar heads are tilted), the ratio E^*_t/E^*_{-t} is about 0.25. This is calculated using simple electrostatic theory of point charges and assuming the distance of closest approach is taken to be the order of d, the dipole length. This is a reasonable length if we consider that at *ca.* 10 Å one must start to remove the "tightly bound water" (e.g., Wilkinson, Morowitz & Prestegard, 1977; Small, 1967), which may in fact be difficult (McAlister *et al.*, 1978; Fuller, Rand & Parsegian, 1979). Compared with the experimental barrier height ratio of 0.22 for DPPE and 0.2 for DMPE, the calculated ratio of 0.25 is reasonable. The calculated ratio of 0.25 gives a $K_{eq} = 10^{+10}$ M^{-1} which is within an order of magnitude of the experimental K_{eq}.

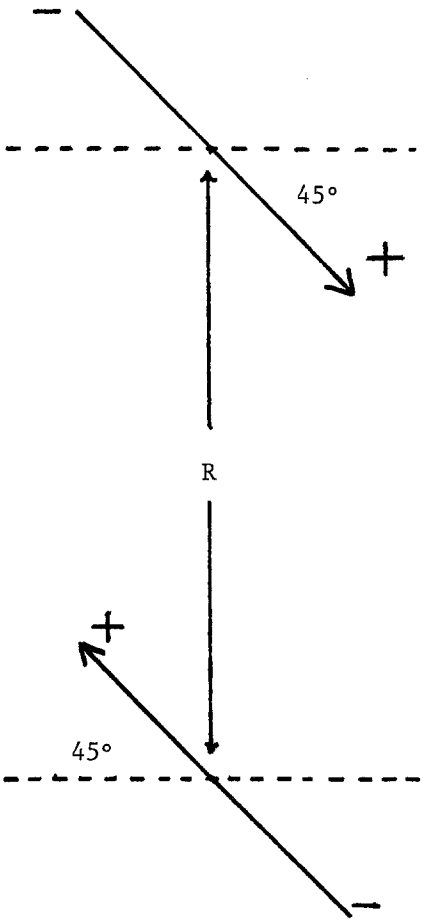

Fig. 9. Two macroscopic dipoles are shown. Each dipole is 45° off the horizontal and has length d. R is the distance between the centers of the dipoles and the arrow on each dipole represents its direction

As the PC molar fraction in the PE/PC membranes increases, the K_{eq} decreases as shown in Table 2. Two models might explain this decrease, (i) the PC forms patches, and (ii) the PE and PC randomly mix. Based on previous studies using 1-anilino-8-naphthalenesulfonate (ANS^-) binding to the membrane, we expect the random mixing model (Haynes & Simkowitz, 1977) to be the correct one. Furthermore, if model i was correct then both K_A and N_{eff} would be independent of the PC content of the vesicle. This follows from the fact that the environment of the PE would be unchanged and both K_A and N_{eff} are indicators of changes in the PE environment. This is not the case (Table 2). In the random mixing model the PC could act stearically to disrupt correlations between the

dipoles and thereby reduce the quantity E^*_{-t} (decrease K_{eq}). It is interesting that for PC molar fractions per vesicle greater than 0.4 we could not induce aggregation. A patching model cannot explain this, but the disruption of intervesicular dipole correlations, due to the PC, in the random mixing model could.

Equilibrium Constant for the Protonation of PE; K_A

As previously mentioned, evidence for the mixing of lipids is the decreasing trend of K_A (Table 2) with increasing PC concentration per vesicle. The apparent pK_a for pure PE vesicles is (*cf.* Table 2) about 7.2. Earlier experiments on PE monolayers (Papahadjopoulos, 1968) performed by microelectrophoresis indicate that the pK_a is no less than 6.5.

One explanation for the decreasing trend of K_A is that the charge density, σ, of the interacting region decreases due to PC displacing PE from the interaction area. Since the dominant ionic species in our experiments is the 0.01-M Tris, the square of the charge density is proportional to K_A (*cf.* Haynes, 1974; Haynes & Simkowitz, 1977). This implies

$$K_A^{PC} = (1 - f_{PC})^2 K_A. \tag{13}$$

Table 3 lists the calculated K_A^{PC} values compared with the parameter values. These calculated values indicate that the magnitude and trend of the pK_a is explained by the effect of PC to dilute out the PE^- and reduce its effect on the local H^+ concentration. The lower K_A for 100% DMPE

Table 3. The equilibrium constant for protonation: K_A

Lipid	%PE	Ratio	K_A (M^{-1})	K_A^{GC} (M^{-1})
DPPE	100	–	1.52×10^7	1.52×10^7
	87	2:1	9.49×10^6	8.3×10^6
		1:1		1.15×10^7
DMPE	100	–	6.32×10^6	6.32×10^6
	80	2:1	1.4×10^6	2.28×10^6
		1:1		4.04×10^6

The column ratio represents the distribution ratio $PE_{inside}/PE_{outside}$. K_A is the value obtained using Eq. (10) of the text to fit the data, and K_A^{GC} is the value calculated from Gouy-Chapman screening according to

$$K_A^{GC} = (1 - f_{PC})^2 \cdot K_A(100\%)$$

($K_A = 6.3 \times 10^6$ M^{-1}) compared with K_A for 100% DPPE ($K_A = 1.52 \times 10^{-7}$ M^{-1}) can be explained by the differences in chain length and phase transition temperatures.

N_{eff}: The Number of PE Molecules in the Interaction Area

The quantity N_{eff} represents the number of lipids per unit interaction area that must be protonated in order that aggregation can occur. Table 2 indicates that N_{eff} decreases with increasing PC/PE ratios per vesicle. It is the purpose of this section to explain the magnitude of N_{eff} and why PC causes N_{eff} to decrease.

If one takes the value of N_{eff} for 100% PE as an indicator of the degree of protonation of the PE necessary to promote aggregation; then, on an average, each PE molecule in the interaction area must be approximately 90% protonated for aggregation to occur (assuming 10 lipids per interaction area[2]). This estimate can be increased if the vesicle size increases, the interaction area increases, or both. For the rest of this section we assume that for aggregation to occur every PE molecule must, on an average, be protonated to 90% of its full charge.

When PC is mixed with PE the number of PE molecules per interaction area must decrease by virtue of PC excluding some PE from the interaction area. If f_{PC} is the fraction of PC on the outer surface of the vesicle, then N_{eff} is given by

$$N_{\text{eff}} = 10 \times (1 - f_{\text{PC}}) \times (0.9). \tag{14}$$

Using Eq. (14) to calculate N_{eff} gives the results in Table 4.

The parameter values obtained from the fit to the data by Eq. (14) are in good agreement with the values calculated from microscopic considerations. The effect of introducing PC into the vesicle system is different than changing the average degree of protonation of the PE. This is because when PC is added the repulsion is dipolar in nature, while the increasing of negative charge per PE would be due to electrostatic charge repulsion. The experiments done with Ca^{2+} in the results section are a

[2] The 10 lipids/interaction area is calculated by assuming an average vesicle radius of *ca.* 150 Å (Stollery & Vail, 1977; Hauser & Phillips, 1973) and that each lipid occupies 60 Å^2. The size of the interacting region is determined by including those lipids whose dipole-dipole interaction with their partner lipid on the apposing vesicle is greater than 50% of the dipole-dipole interaction for the lipids at closest approach (*ca.* an angle of 5° measured from the vesicles, center-to-center line).

Table 4. The effective interaction area: N_{eff}

%PE	N_{eff}	N^c_{eff}	
		2:1	1:1
100	9.6	[9.0]	[9.0]
87	7.0	6.7	7.8
80	3.8	5.4	7.2

N_{eff} represents the fit values from the data, and N^c_{eff} is the calculated value according to

$$N^c_{eff} = 10 \times (1 - f_{PC}) \times 0.9.$$

The 0.9 for 100% PE is bracketed because it is an approximate value. Whether the lipid is above its T_c or below, it has not been considered, because the number of lipids per interaction area is approximately 10 for both DPPE and DMPE.

The distribution ratios (2:1 and 1:1) provide the two columns below N^c_{eff}. The 2:1 for our case puts all the PC on the outside of the vesicle, while the 1:1 ratio puts equal amounts on the inside and outside.

further indication that a net negative charge can exist on the PE vesicles at pH 7.

A number of authors (Parsegian & Gingell, 1972; Nir, 1976) have explained how the negative surface charge can be counterbalanced by the electrodynamic van der Waals force. Nir (1976) has predicted that vesicles with a charge density exceeding one tenth of the electronic charge of the phospholipid molecule have a small probability of approaching within 25 Å of each other. Similarly, Parsegian and Gingell (1972) show that for a high surface charge density (600 Å^2/charge) both the electrostatic and electrodynamic energies are on the order of 10^{-1} erg/cm^2 for a 10-Å separation.

It is important to note that, because of the high protonation requirement of the lipids in the interacting region (90%) for aggregation, the transition temperature of the interacting region must be close to T_c for fully protonated lipids. Introduction of PC into the interacting region will lower the T_c (Blume & Ackermann, 1974), but not significantly for the ratios we have used.

Biological Significance of PE

It can be inferred from our results that PE plays a significant role in determining the propensity of membranes to stick together. A possible

regulatory effect is indicated by the fact that the pH necessary to produce substantial aggregation (i.e., $\alpha = 0.5$) is close to physiological pH (*ca.* 7) for the lipid systems we considered. This supports our reasoning about the role of PE in Ca^{2+} induced chromaffin granule aggregation (Morris *et al.*, 1978). We suggest that mixing other lipids with PE tend to regulate K_{eq} and the apparent pK_a (pH at 50% ionization). Our results on PE indicate that lipids in the liquid crystalline state tend to have lower apparent pK_a than lipids in the crystalline state. This result is in agreement with work done on PS vesicles (MacDonald, Simon & Baer, 1976) and indicate that the lipid chain length might play a significant role in adjusting the apparent pK_a so that aggregation is favored at physiological pH. If this hypothesis is true, systems undergoing aggregation processes should contain predominantly long-chain (14, 16, 18 carbons) saturated PE molecules.

Conclusions

In this paper we have presented experimental results on the aggregation of PE and PE/PC mixed vesicles as a function of pH. An equilibrium equation was derived whose fit parameters could be explained in terms of simple electrostatic arguments. These parameters are decreasing functions of the mole fraction of PC per vesicle. From this theory a basic picture of PE aggregation phenomena and the possible significance of PE in biological systems was considered. Some of the new results were that aggregation occurs due to intervesicular head-to-tail binding of PE molecules, that the K_A for the PE is explicable in terms of Gouy-Chapman theory, and that a minimum interaction area is necessary for these vesicles to aggregate. The interpretation of the experimental results indicate that at pH 7 the PE vesicles carry a net negative charge. Furthermore, the data indicate that PE and PC mix randomly. This interpretation is different from those for Ca^{2+} induced aggregation of PA/PC vesicles (Lansman & Haynes, 1975) where it was found that exclusion of PC from the interaction region is necessary for aggregation. Biologically, we have interpreted the results to indicate that when the T_c (phase transition temperature) of the lipids is above the temperature of the system, aggregation at physiological pH is favored. The results of this study lead us to believe that PE plays an important role in the aggregation reactions of secretion granules (Morris *et al.*, 1978) in particular

and suggest that PE may act as a modular at membrane-membrane contact in general.

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Note Added in Proof: The apparent pK_a determined here is for 90% protonation. Using the Gouy-Chapman theory to calculate its value for 50% protonation gives 8.1 in agreement with our recent potentiometric titration experiments.

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