

Studies on the Mechanism of Membrane Fusion: Evidence for an Intermembrane Ca^{2+} -Phospholipid Complex, Synergism with Mg^{2+} , and Inhibition by Spectrin[†]

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ABSTRACT: The interaction of Ca^{2+} and Mg^{2+} with phosphatidylserine (PS) vesicles in 0.1 M NaCl aqueous solution was studied by equilibrium dialysis binding, X-ray diffraction, batch microcalorimetry, kinetics of cation-induced vesicle aggregation, release of vesicle contents, and fusion. Addition of either cation causes aggregation of PS vesicles and produces complexes with similar stoichiometry (1:2 cation/PS) at saturating concentrations, although the details of the interactions and the resulting complexes are quite different. Addition of Ca^{2+} to PS vesicles at $T \geq 25^\circ\text{C}$ induces the formation of an "anhydrous" complex of closely apposed membranes with highly ordered crystalline acyl chains and a very high transition temperature ($T_c > 100^\circ\text{C}$). The formation of this complex is accompanied by a release of heat (5.5 kcal/mol), rapid release of vesicle contents, and fusion of the vesicles into larger membranous structures. By contrast, addition of Mg^{2+} produces a complex with PS which is much

more hydrated, has no crystallization of the acyl chains at $T \geq 20^\circ\text{C}$, and has comparatively little fusion. Studies with both Ca^{2+} and Mg^{2+} added simultaneously indicate that there is a synergistic effect between the two cations, which results in an enhancement of the ability of Ca^{2+} to form its specific complex with PS at lower concentrations. The presence of the erythrocyte protein "spectrin" inhibits this synergism and interferes with the formation of the specific PS/Ca complex. It also inhibits the fusion of PS vesicles. It is proposed that the unique PS/Ca complex, which involves close apposition of vesicle membranes, is an intermembrane "trans" complex. We further propose that such a complex is a key step for the resultant phase transition and fusion of PS vesicles. By contrast, the PS/Mg complex is proposed to be a "cis" complex with respect to each membrane. The results are discussed in terms of the mechanism of membrane fusion.

The importance of the interactions of divalent cations with acidic phospholipids in biological membranes is becoming increasingly apparent. It is now well documented that Ca^{2+} plays a central role in membrane fusion phenomena such as cellular secretion and acetylcholine release in the presynaptic nerve endings (Poste & Allison, 1973; Rubin, 1974; Douglas, 1975). The effects of divalent cations on the thermotropic properties of acidic phospholipids could be an essential component of such phenomena, as well as other membrane-associated activities. We have, therefore, undertaken extensive studies of the effects of Ca^{2+} and Mg^{2+} on the properties of acidic phospholipids in various model systems using several diverse techniques (Newton et al., 1978; Papahadjopoulos et al., 1976, 1977; Jacobson & Papahadjopoulos, 1975). The emphasis in our recent studies has centered on the contrasting interactions of Ca^{2+} and Mg^{2+} with phosphatidylserine (PS),¹ their induction of lipid phase separations, and their possible relation to the mechanism of membrane fusion (Newton et al., 1978; Papahadjopoulos et al., 1977, 1978).

We have previously shown that the addition of Ca^{2+} (≥ 1 mM) to PS vesicles causes aggregation and fusion of the vesicles accompanied by a marked increase in vesicle permeability (Papahadjopoulos & Bangham, 1966; Papahadjopoulos et al., 1977). Fusion of the vesicles eventually results in the formation of cochleate lipid cylinders (Papahadjopoulos et al., 1975) of defined stoichiometry ($\text{Ca}^{2+}/\text{PS} = 1:2$) and with a transition temperature (T_c) shifted to a very high value ($>70^\circ\text{C}$) (Jacobson & Papahadjopoulos, 1975).

Considerably higher concentrations of Mg^{2+} (≥ 3 mM) are required to induce aggregation of PS vesicles, which is accompanied only by limited fusion (Papahadjopoulos et al., 1977). At these concentrations Mg^{2+} binds to a similar extent as Ca^{2+} but shifts the T_c of PS upward by only about 10°C (from $\sim 8^\circ\text{C}$ in 100 mM NaCl to 18°C).

X-ray diffraction studies, discussed more fully below, reveal striking structural differences in the PS bilayers following Ca^{2+} and Mg^{2+} addition (Newton et al., 1978). These studies along with recent NMR data (Hauser et al., 1977) indicate that Ca^{2+} (but not Mg^{2+}) addition results in a close apposition of the bilayers, essentially free of interlamellar water, and a highly ordered acyl chain packing. Such close apposition raises the possibility of Ca^{2+} interaction with PS head groups from the two apposed membranes ("trans" complex). Such an intermembrane complex would be radically different from cation binding to PS head groups in the plane of each bilayer ("cis" complex). In the former case, the formation of the complex would of necessity be preceded by close apposition of the PS bilayers. If true, this requirement for formation of the PS/Ca complex has important implications for the role of Ca^{2+} in the fusion and phase separation of acidic phospholipids and their possible relationship to in vivo mechanisms of membrane fusion.

In this paper we present experiments that investigate the requirement of close apposition and possible "trans" complex formation for the Ca^{2+} -induced crystallization of the acyl chains, increase in vesicle permeability, and fusion of PS vesicles.

Materials and Methods

Phosphatidylserine (PS) was purified from bovine brain in this laboratory as previously described (Papahadjopoulos et

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¹ Abbreviations used: PS, phosphatidylserine isolated from bovine brain; CF, carboxyfluorescein; DSC, differential scanning calorimetry; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; IMP, intramembranous particles.

al., 1977; Papahadjopoulos & Miller, 1967). It was washed with EDTA to remove metal impurities and was kept as a solution in chloroform in sealed ampules under nitrogen. Spectrin was isolated (Fairbanks et al., 1971) from freshly drawn blood of human volunteers and was kindly provided by Dr. C. Y. Jung (V.A. Hospital, Buffalo, NY). The solutions of spectrin were dialyzed overnight at 4 °C against the standard NaCl buffer and then centrifuged at 84000g for 30 min to remove any large aggregates. It was kept at 4 °C and was used within 24–48 h. NaDodSO₄ gel electrophoresis (Laemmli, 1970) indicated the presence of the two spectrin bands with a minor band at 47000 molecular weight. Human albumin was obtained from Miles Laboratories, Inc. Carboxyfluorescein (CF) was obtained from Eastman Kodak (no. 9952) and was recrystallized from ethanol/water (Blumenthal et al., 1977). All other chemicals were reagent grade. Water was twice distilled, the second time in an all-glass apparatus.

Dispersions of multilamellar vesicles and sonicated unilamellar vesicles of PS were prepared as described before (Papahadjopoulos et al., 1977) in a standard buffer containing 100 mM NaCl, 2 mM L-histidine, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), and 0.1 mM EDTA, adjusted to pH 7.4 and a concentration of 2–6 μmol of phospholipid per mL. Carboxyfluorescein (CF)-containing vesicles were prepared by hydration and sonication of PS in a solution of 100 mM CF, 0.1 mM EDTA, and 1/10 (v/v) of the standard buffer, adjusted to pH 7.4. The vesicles were separated from free CF by passage through a Sephadex G-75 column (1.0 \times 20 cm), equilibrated with the standard (0.1 M NaCl) buffer, and subsequently stored on ice. For the experiments presented in Figures 3 and 5, a population of larger size vesicles containing CF was isolated from the sonicated preparations following separation on the Sephadex column (12 μmol of PS in 1.8 mL) by modifications of the differential centrifugation procedure of Barenholz et al. (1977). The vesicles were centrifuged in a Beckman SW 50.1 rotor at 4 °C as follows: (a) 84000g for 30 min; (b) the supernatant was collected and centrifuged at 133000g for 60 min; (c) the pellet was collected, resuspended in 1.2 mL, and centrifuged at 133000g for 60 min; (d) the pellet was removed and resuspended in a small volume of buffer (yield \sim 15%). These vesicles have an average size of \sim 550 Å compared with a broad size distribution and an average diameter of \sim 300 Å for the initial sonicated but uncentrifuged preparations, as measured by dynamic light scattering (Day et al., 1977), courtesy of Drs. E. P. Day and J. T. Ho, SUNY, Buffalo, NY.

Binding studies were carried out by equilibrium dialysis of sonicated PS vesicles dialyzed against the standard NaCl buffer (without EDTA) containing various concentrations of Ca^{2+} or Mg^{2+} for 6 h at 37 °C (Newton et al., 1978). The samples were at apparent equilibrium after 6 h since dialysis of some samples for 12 h gave identical results. Many of the binding experiments were done with vesicles in the presence of the ionophore X-537A (courtesy of Hoffmann-La Roche). In these experiments the ionophore was mixed with the phosphatidylserine in chloroform at a molar ratio of 1 ionophore:100 PS before evaporation. Dispersion and sonication of these vesicles were carried out as usual. At concentrations of $\text{Ca} < 1$ mM and $\text{Mg} \leq 3$ mM, the concentration of PS used for all binding experiments was 10 $\mu\text{mol}/\text{mL}$, and the amount of cation associated with lipid was determined by comparing aliquots from the dialysis bag and the bulk solution. At concentrations of $\text{Ca} \geq 1$ mM and $\text{Mg} > 3$ mM, the concentration of PS used was 2 $\mu\text{mol}/\text{mL}$. Under these conditions the cation-lipid complex formed aggregates and pelleted

(>95%) when centrifuged at 100000g for 30 min. The pellets were weighed wet and after high vacuum drying over phosphorus pentoxide. The difference between the wet and dry weight was used to give an estimate of the bulk solution associated with the pellet. The pellets were analyzed for lipid phosphate (Fiske & Subbarow, 1925) and cations which were determined with a Perkin-Elmer 370 A atomic absorption spectrophotometer. Ratios of PS to Ca or Mg were calculated after adjusting for the amount of cations in the bulk solution in the pellet.

Phase transitions were detected with a Perkin-Elmer DSC-2 differential scanning calorimeter as before (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978). X-ray diffraction was conducted using Cu $\text{K}\alpha$ radiation in either a quartz monochromatized Guinier camera or a Ni-filtered Franks camera, as before (Newton et al., 1978). Batch microcalorimetry experiments were conducted with a LKB 2107 batch microcalorimeter (Papahadjopoulos et al., 1978). Fluorescence of the vesicles containing carboxyfluorescein was measured with an Aminco-Bowman spectrofluorimeter (excitation, 490 nm; emission, 550 nm) using a Corning cut-off filter (no. 3-68, \sim 520 nm). Complete release of the carboxyfluorescein was obtained by the addition of Triton X-100 (\sim 0.05% v/v). Light scattering (at 90°) was measured with the same instrument with excitation and emission both set to 400 nm.

The binding of spectrin to PS vesicles was followed by density gradient centrifugation on discontinuous sucrose density steps 1.01 (2.5%), 1.11 (25%), 1.13 (30%), 1.15 (35%), and 1.18 (40%) g/mL. The vesicles were mixed with the protein (2:1 w/w lipid to protein) and dialyzed for 3 h at 22 °C against standard buffer containing 1 mM Ca^{2+} or 3 mM Mg^{2+} . The contents of the dialysis bags were then placed on the sucrose gradient and centrifuged at 58000g for 16 h at 20 °C in a Beckman SW 50.1 rotor. The gradient steps were then removed and dialyzed against distilled water, and the fractions were analyzed for protein (Lowry et al., 1951) and lipid phosphate (Fiske & Subbarow, 1925).

Results and Discussion

Binding of Ca^{2+} , Mg^{2+} , and Ca^{2+} with Mg^{2+} to Phosphatidylserine. Preliminary data on the binding of Ca^{2+} and Mg^{2+} to sonicated PS vesicles prepared in 0.1 M NaCl solution were presented recently (Newton et al., 1978). These studies showed that both Ca^{2+} and Mg^{2+} were associated with PS vesicles to a much higher extent than would be expected from simple electrostatic double-layer screening. Moreover, Ca^{2+} was found to bind to PS much more strongly than Mg^{2+} (Newton et al., 1978). A more detailed study of Ca^{2+} and Mg^{2+} binding to PS vesicles is presented in Figure 1. In addition we present data on the amount of Ca^{2+} and Mg^{2+} bound simultaneously at various Ca^{2+} concentrations in the presence of a relatively high Mg^{2+} concentration (5 mM). As will be discussed below, aggregation occurs with either Ca^{2+} or Mg^{2+} at concentrations close to or above those required for a ratio of 0.41 (M^{2+}/PS). The data from the binding of each ion alone (in the presence of 0.1 M NaCl) indicate that PS has a higher affinity for Ca^{2+} than for Mg^{2+} . The apparent binding constants (K_a) calculated from a Scatchard plot (Scatchard, 1949) of the data points summarized in Figure 1 give values of $\sim 3.9 \times 10^3 \text{ M}^{-1}$ for Ca^{2+} and $\sim 1.6 \times 10^3 \text{ M}^{-1}$ for Mg^{2+} . The apparent binding constants are, of course, higher numerically than the intrinsic binding constants (35 M^{-1} for Ca^{2+} and 20 M^{-1} for Mg^{2+} , courtesy of S. Nir) calculated on the basis of a modified Gouy-Chapman equation (Nir et al., 1978) due to the influence of the double layer as

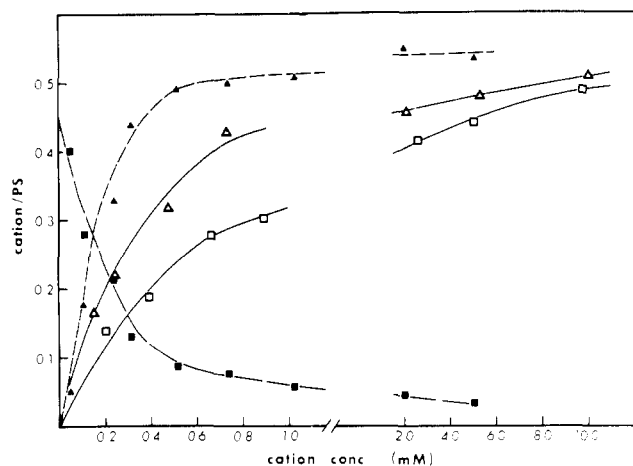


FIGURE 1: Binding of Ca^{2+} , Mg^{2+} , and Ca^{2+} with 5 mM Mg^{2+} to sonicated PS vesicles. For experimental details see Materials and Methods. (Δ) Ca^{2+} ; (\square) Mg^{2+} ; (\blacksquare) Ca^{2+} in the presence of 5 mM Mg^{2+} . For simplicity only a few representative points for the Ca^{2+} and Mg^{2+} binding curves are shown. Most of the points are from samples with ionophore, but some points from samples without ionophore are included. Vesicles without ionophore are not permeable at low cation concentrations (see text, below); ratios for such samples were therefore calculated with the assumption that 60% of the lipid was exposed to the bulk solution. At any given bulk cation concentration, no significant difference was found between calculated ratios for samples with and without ionophore. The apparent binding constants (K_a) were calculated by a least-squares fit of all the binding data (43 points for Ca^{2+} , 34 points for Mg^{2+}) in a Scatchard plot (Scatchard, 1949). The K_a for Ca^{2+} at the 95% confidence level is $3.9 \pm 1.0 \times 10^3 \text{ M}^{-1}$ with a correlation coefficient $R = 0.84$. For Mg^{2+} , the K_a is $1.6 \pm 0.2 \times 10^3 \text{ M}^{-1}$ at the 95% confidence level ($R = 0.97$).

discussed earlier (Newton et al., 1978). The observed values for Mg^{2+} alone are approximately twofold greater than those reported previously (Newton et al., 1978). The values presented in Figure 1 are considered to be more accurate due to the use of a fivefold greater lipid concentration in the present experiments, in order to reduce the required corrections from the bulk phase in equilibrium dialysis.

The most striking result in Figure 1 is the absence of competition between Ca^{2+} and Mg^{2+} when both ions are present simultaneously. At low Ca^{2+} concentrations ($<1 \text{ mM}$), Ca^{2+} binding is enhanced in the presence of 5 mM Mg^{2+} . For example, at 0.2 mM Ca^{2+} concentration there is 0.33 Ca^{2+}/PS and only 0.21 Mg^{2+}/PS in spite of the predominance of Mg^{2+} in the bulk phase by 25-fold. Under these conditions the amount of Ca bound to PS is considerably more than that which is bound in the absence of Mg^{2+} . These results are not what would be expected if both Ca^{2+} and Mg^{2+} were bound to equivalent sites. However, the results can be rationalized if we postulate that, when Mg^{2+} is present at low concentrations of Ca^{2+} , a new binding mode becomes possible, which is specific for Ca^{2+} , with a very high binding constant. As will be discussed below, this new binding mode, occurring only under conditions when vesicles aggregate, can be equated with the formation of a characteristic intermembrane Ca^{2+} complex observed by X-ray diffraction. There is some suggestion for a break in the Ca^{2+} binding curve between 0.5 and 0.7 mM which may be correlated with the observed difference between vesicle aggregation at these two concentrations and the possible contribution of a second binding constant expressed only at the higher concentrations.

X-ray Diffraction Studies with Phosphatidylserine: Indications of an Anhydrous, "Trans" Complex of PS and Ca^{2+} . A summary of our previous and some more recent X-ray

Table I: Summary of X-ray Diffraction Spacings of Phosphatidylserine

lipid sample ^b	X-ray diffraction spacings ^a (Å)	
	low temp, 5 °C	high temp, 25 °C
(a) PS/Na (0.1 M)		78d (4.6d)
(b) PS/Na (1 M)	71 (4.2)	66 (4.6d)
(c) PS/Na (dry)		60 (4.2)
(d) PS/Mg	67 (4.2)	53 (4.6d)
(e) PS/Mg (dry)	60 (4.2)	60 (4.2)
(f) PS/Ca	53 (4.1, 4.5)	53 ^c (4.1, 4.5)
(g) PS/Ca (dry)	53 (4.1, 4.5)	53 (4.1, 4.5)
(h) PS (Ca + Mg)		53 (4.1, 4.5)

^a The first figure indicates the lamellar repeat spacing which includes any water space between the bilayers. The figure in parentheses is the high angle spacing which arises from the packing of the acyl chains. d indicates a diffuse diffraction. ^b Samples were prepared as follows: (a) PS hydrated in excess standard buffer at pH 7.4; (b, d, f) PS hydrated in excess standard buffer containing 1 M NaCl, 5–20 mM Mg^{2+} , and 5–20 mM Ca^{2+} , respectively, or sonicated PS vesicles precipitated by dialysis against the same buffers; (c) Na⁺ salt of PS dried from chloroform, before hydration; (e, g) same as d, f except followed by high vacuum drying; (h) sonicated PS vesicles dialyzed (12 h, 25 °C) against buffer containing 0.05 mM Ca^{2+} and 5 mM Mg^{2+} . ^c No change, even at 60 °C.

diffraction studies of bovine brain phosphatidylserine (PS) under various conditions is shown in Table I. PS suspended in 100 mM NaCl and pH 7.4 at 25 °C results in a very diffuse lamellar spacing centered about 78 Å (Papahadjopoulos & Miller, 1967), with a diffuse 4.6-Å high-angle line characteristic of fluid acyl chains (Shipley, 1973). The thickness of the bilayer itself under similar conditions has been calculated to be approximately 40 Å (Shipley, 1973). Slow controlled hydration with low salt can result in even greater lamellar spacings ($>100 \text{ Å}$) due to the repulsive forces between the bilayers associated with the high negative surface charge density of the membranes (Shipley, 1973). PS membranes in high salt (1 M NaCl) sufficient to precipitate PS vesicles have a shorter lamellar spacing of 66 Å, presumably because the electrostatic repulsive forces between vesicles are reduced. At 5 °C, below the phase transition temperature, the lamellar spacing is somewhat greater (71 Å) and a sharp 4.2-Å high-angle diffraction appears, which is characteristic of hexagonally packed but rotationally disordered acyl chains (Shipley, 1973). The thickness of the bilayer under these conditions is probably about 60 Å as this is the spacing obtained when PS is dried down from CHCl_3 , before hydration, and which also has a 4.2-Å high-angle spacing. PS membranes in MgCl_2 (5–20 mM) are somewhat similar to those in high NaCl, but with reduced lamellar spacings both above (53 Å) and below (67 Å) the phase transition temperature. At these Mg^{2+} concentrations, most of the surface charge density is neutralized by Mg^{2+} binding (Figure 1). However, considerable water remains between the membranes as indicated by comparison with the actual bilayer widths estimated above and the altered diffraction pattern (60 Å and 4.2 Å) obtained after high-vacuum drying.

In marked contrast to the above samples, PS membranes exposed to Ca^{2+} have a very short lamellar spacing of 53 Å and sharp 4.1- and 4.5-Å high-angle diffraction spacings indicative of a highly ordered acyl-chain packing, but which do not correspond to any known subcell packing. The short lamellar spacing would appear to leave little room for interlamellar water, a hypothesis which is supported by the lack of change in the X-ray diffraction pattern after high-vacuum drying. The absence of free water in the complex between PS and Ca^{2+} formed under these conditions has also been sug-

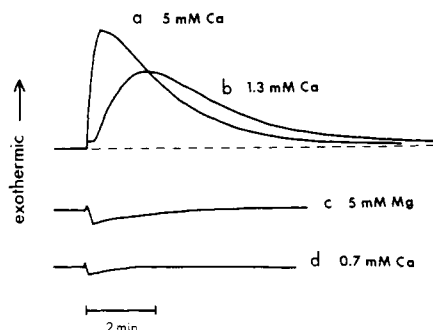


FIGURE 2: Microcalorimetric traces of heat flow upon mixing of Ca^{2+} and Mg^{2+} with PS vesicles. Buffer (4 mL) containing the appropriate concentrations of Ca^{2+} or Mg^{2+} was placed in one-half of the sample and reference cells, and 2 mL of the buffer or buffer and PS vesicles ($1\text{--}2\text{ }\mu\text{mol}$ of PS) was placed in the other half. After temperature equilibration ($25\text{ }^{\circ}\text{C}$, $\sim 1\text{ h}$), the samples were mixed and heat flow was recorded. Calibration standards (15-s electrical heating) were run after each sample (time course of heat flow is similar to that of 5 mM Ca^{2+}) and agreed $\pm 5\%$. A small exothermic reaction ($\sim 0.6\text{ kcal/mol}$ not shown) was observed when PS vesicles were mixed with buffer alone but its origin is not understood at present. Concentrations indicated are those after mixing assuming no binding to the PS. Enthalpies of reaction were (a and b) $5.5 \pm 0.5\text{ kcal/mol}$, (c) $\sim 0.2\text{ kcal/mol}$, and (d) $\sim 0.1\text{ kcal/mol}$.

gested from NMR studies (Hauser et al., 1977). Heating to $60\text{ }^{\circ}\text{C}$ has no effect on the PS/Ca structure, in confirmation of the absence of a phase transition in differential scanning calorimetry (DSC) studies (Jacobson & Papahadjopoulos, 1975). The same type of complex is formed in the presence of Ca^{2+} (0.05 mM) and Mg^{2+} (5 mM) as shown in Table I (line h). This remarkable observation will be discussed in detail below.

These X-ray diffraction results indicate that Ca^{2+} binding to PS membranes can result in the formation of a unique and entirely different complex from that formed with Mg^{2+} . We propose that such a complex could involve the chelation of Ca^{2+} by phosphates and carboxyl groups from the apposed bilayers in an intermembrane complex where water is either absent or participating stoichiometrically in the complex. We call such a complex a trans complex. By comparison, we propose that the Mg^{2+} binds to only one bilayer surface in an arrangement which could be called a cis complex, with a thin layer ($\sim 7\text{ \AA}$) of water still separating the bilayers. As will be discussed below, we have obtained evidence indicating that the formation of the trans Ca^{2+} complex is a crucial step for the subsequent fusion of the vesicles.

A Ca^{2+} -Induced Isothermal Phase Transition of PS Observed with Batch Microcalorimetry. The results of our X-ray diffraction and DSC studies indicate that Ca^{2+} , but not Mg^{2+} , can induce an isothermal phase transition in PS membranes, from fluid to a solid crystalline acyl-chain packing, at temperatures above $25\text{ }^{\circ}\text{C}$. Microcalorimetry is well suited to measuring the enthalpies associated with isothermal phase transitions. Therefore we investigated the effects of Ca^{2+} and Mg^{2+} addition to sonicated PS vesicles at $25\text{ }^{\circ}\text{C}$ in a batch microcalorimeter. A preliminary account of some of these results has been published (Papahadjopoulos et al., 1978).

As shown in Figure 2, the addition of Mg^{2+} (5 mM) or low Ca^{2+} concentrations (0.7 mM) was observed to be slightly endothermic. However, the addition of Ca^{2+} concentrations ($1.3\text{--}5\text{ mM}$) sufficient to cause fusion, disappearance of the phase transition (in DSC), and formation of the crystalline PS/ Ca^{2+} membranes (in X-ray diffraction) was highly exothermic (5.5 kcal/mol). The results obtained at low Ca^{2+} or high Mg^{2+} concentrations indicate that cation binding and aggregation of the vesicles do not have large enthalpies of

reaction. However, the enthalpy of reaction observed at higher Ca^{2+} concentrations can be mostly accounted for by the release of heat associated with crystallization of the acyl chains induced by Ca^{2+} under these conditions. The enthalpy associated with melting of the chains in the PS/ Ca^{2+} complex is still unknown,² but the value is probably greater (Van Dijck et al., 1975) than that of the enthalpy of the PS transition in NaCl (4.5 kcal/mol).

A relatively slow release of heat ($t_{1/2} \approx 2\text{ min}$) was observed when 1.3 mM Ca^{2+} was added to the vesicles. The observed delay could be related to residual electrostatic repulsion between vesicles and the kinetics of vesicle aggregation, since binding studies indicate that PS might not be completely neutralized at this Ca^{2+} concentration (Figure 1). This point is of considerable interest to us since it suggests that the release of heat might not occur until a close apposition between membranes is achieved. In such a case, the release of heat would be kinetically limited by vesicle-vesicle contact and would be concomitant with the formation of the closely apposed anhydrous Ca^{2+} complex observed by X-ray diffraction. We plan to further investigate this question by studying the kinetics of heat release at different vesicle concentrations.

Kinetics of Aggregation and Increased Permeability Induced by Ca^{2+} . The results presented above prompted us to investigate whether the dramatic increase in the permeability of PS vesicles induced by Ca^{2+} (Papahadjopoulos et al., 1977) is dependent on close contact between membranes and aggregation of the vesicles. The concentrations of Ca^{2+} required for the permeability increase correlate closely with those causing aggregation, the dramatic shift in the phase transition temperature, formation of the crystalline PS/Ca complex, and fusion of the vesicles (Newton et al., 1978; Papahadjopoulos et al., 1977). However, the relative kinetics of these processes and the possible importance of vesicle-vesicle contact for events other than aggregation and fusion were unclear. Therefore, it was of considerable interest to determine if Ca^{2+} addition to PS vesicles could cause a release of contents in the absence of extensive aggregation or fusion. Previous evidence suggested that a Ca^{2+} -induced crystallization of the acyl chains could be expected to result in a marked permeability increase (Papahadjopoulos et al., 1973) even in the absence of vesicle-vesicle contact and fusion. Therefore, we investigated the kinetics of Ca^{2+} -induced permeability increase and its relationship to vesicle aggregation and concentration.

The release of vesicle contents was followed conveniently by the enhancement of fluorescence intensity that occurs upon release and dilution of carboxyfluorescein (CF) initially encapsulated within the vesicles at high (self-quenching) concentrations (Blumenthal et al., 1977; Weinstein et al., 1977). This technique permitted kinetic studies at very low vesicle, but high Ca^{2+} , concentrations and in very short time periods. $^{22}\text{Na}^{+}$ permeability experiments described previously (Papahadjopoulos & Watkins, 1967) are more suitable for high vesicle concentrations and low efflux rates. The kinetics of aggregation were followed qualitatively by increases in light scattering under conditions identical with those of CF release.

As shown in Figure 3, the kinetics of aggregation and CF release following addition of Ca^{2+} were both markedly dependent on Ca^{2+} concentrations with an apparent threshold near 1 mM Ca^{2+} . A similar threshold was observed in ^{22}Na permeability studies (Papahadjopoulos & Watkins, 1967;

² An irreversible endothermic transition accompanied by partial decomposition and centered at $130\text{ }^{\circ}\text{C}$ has been observed when the PS/Ca complex in 0.1 M NaCl buffer is heated above $100\text{ }^{\circ}\text{C}$ in high pressure pans.

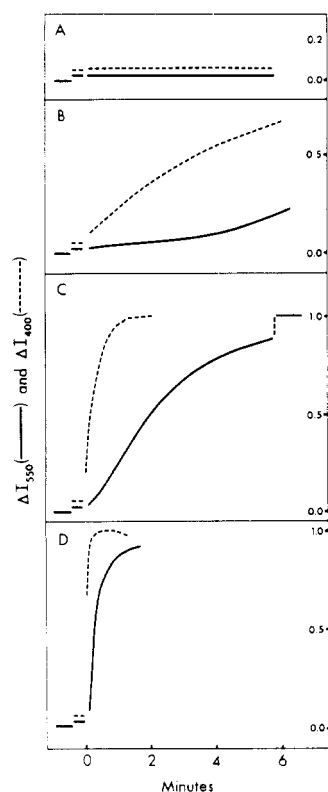


FIGURE 3: Time course of aggregation (---) and release of CF (—) from PS vesicles (20 μ M) after addition of Ca^{2+} . (A) With 0.5, (B) 1, (C) 2, and (D) 5 mM Ca^{2+} . Temperature was 22 °C. Aggregation was measured by the increase in 90° light scattering (ΔI_{400}) and release of CF by the increase in fluorescence intensity (ΔI_{550}).

Papahadjopoulos et al., 1977). Under the conditions of the experiment in Figure 3, it is clear that aggregation precedes CF release, which at 1–2 mM Ca^{2+} shows a considerable delay. The presence and extent of the delay in CF release was found to depend on Ca^{2+} concentration, vesicle concentration, and temperature and is not as yet completely understood. Of more immediate interest is that a significant increase in the rate of CF release (minimal observable rate is $\sim 10\%$ per h) was only observed concomitant to or following an increase in light scattering even at very low vesicle (1 μ M PS) and very high (10 mM) Ca^{2+} concentrations.

The dependence of CF release on vesicle-vesicle collisions was more strongly suggested by the finding that the maximal rate of CF release caused by 2 mM Ca^{2+} was nearly second order in its dependence on vesicle concentration when varied from 0.5 to 50 μ M lipid. The plot of the logarithm of the maximal rate of fluorescence increase ($\Delta I_{550}/\text{min}$) against the logarithm of PS concentration (μ M) gave a straight line with good fit and a slope of 1.8. Similar results were also found when 5 or 10 mM Ca^{2+} was added to the vesicles.³ The results of these experiments indicate, but of course do not prove, that

³ The maximal rate of CF release was determined by following the rate of increase in fluorescence following addition of Ca^{2+} to sonicated PS vesicles containing CF (see Materials and Methods) at 25 °C. The rate was taken from the linear part of the plot of fluorescence intensity vs. time where the slope was maximal. The delay between zero time and the linear increase (1–30 s) depended on vesicle concentration, being more pronounced at the lower range. At low concentrations (0.5 μ M lipid), release was followed for an extended time period (5–10 min), while at higher vesicle concentrations the release was complete within a few minutes or faster, depending on Ca^{2+} concentration. Separate experiments showed that fluorescence per μ mol of CF was linear and that there was essentially no interferences from light scattering due to aggregation of the vesicles under the conditions of the experiment.

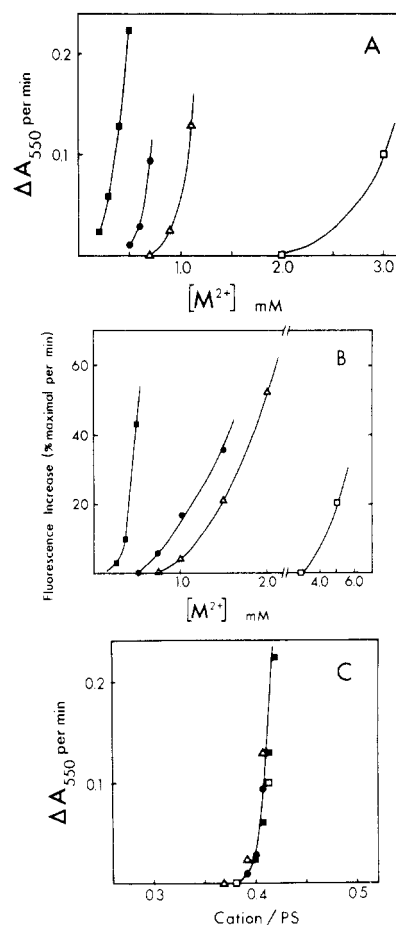


FIGURE 4: The effects of Ca^{2+} , Mg^{2+} , or Ca^{2+} in the presence of Mg^{2+} on aggregation (A) or the permeability (B) of sonicated preparations of PS and the dependence of aggregation on the extent of cation binding (C). (A) The rates shown are estimated from the initial part of the absorbance increase at 550 nm obtained by the rapid addition of 1 mL of buffer containing twice the indicated concentrations of Ca^{2+} and Mg^{2+} to 1 mL of buffer containing the vesicles (500 μ M PS) at 22 °C; (Δ) Ca^{2+} ; (\square) Mg^{2+} ; (\bullet) 1 mM Mg^{2+} + Ca^{2+} ; (\blacksquare) 2 mM Mg^{2+} + Ca^{2+} . (B) The rates shown are estimated from the initial part of the release curve unless the release was biphasic (Ca^{2+} or Ca^{2+} + 1 mM Mg^{2+}) in which case the maximal rate of release was calculated. Release caused by Mg^{2+} alone was incomplete with only 50% increase in fluorescence occurring after addition of 7 mM Mg^{2+} . Release was initiated by addition of small aliquots of Ca^{2+} or Mg^{2+} , with rapid mixing, to the PS vesicles (67 μ M) at 22 °C; (\square) Mg^{2+} ; (Δ) Ca^{2+} ; (\bullet) Ca^{2+} + 1 mM Mg^{2+} ; (\blacksquare) Ca^{2+} + 3 mM Mg^{2+} . (C) The initial rate of aggregation shown in A replotted vs. the extent of cation binding to PS as calculated from the apparent binding constants derived from Figure 1. Cation binding in the presence of both Ca^{2+} and Mg^{2+} was calculated assuming competition between the cations for binding (cis complex). (Δ) Ca^{2+} ; (\square) Mg^{2+} ; (\bullet) 1 mM Mg^{2+} + Ca^{2+} ; (\blacksquare) 2 mM Mg^{2+} + Ca^{2+} .

the Ca^{2+} -induced increase in vesicle permeability requires contact between vesicles, suggesting that it is correlated with the formation of the intermembrane PS/ Ca complex (observed by X-ray diffraction) and Ca^{2+} -induced fusion of PS vesicles. The data tend to exclude the possibility that single vesicles become unstable in the presence of Ca^{2+} , in which case the rate of release should be first order with respect to vesicle concentration. However, further studies under conditions approximating those where slow heat release was observed in the batch microcalorimetry experiments are required to establish in detail the relative kinetics of the permeability increase, heat release, and fusion.

Kinetics of Aggregation and Increased Permeability Induced by Mg^{2+} and Apparent Synergism with Ca^{2+} . The previous experiments indicated that the ability to form close contact

between apposed membranes (i.e., aggregation) and not the extent of metal binding, per se, might be the limiting factor for the formation of the crystalline PS/Ca complex. Therefore it was of interest to examine the role of Mg^{2+} (alone or in the presence of Ca^{2+}) since the presence and binding of Mg^{2+} would be expected to reduce the surface charge density of the PS vesicles and thus enhance the frequency of close contact collisions.

The effects of Ca^{2+} , Mg^{2+} , and Ca^{2+} with Mg^{2+} on PS vesicle aggregation are shown in Figure 4A. The initial rate of increase in optical density at 550 nm (which we consider to reflect aggregation and possible fusion) is markedly dependent on cation concentration with apparent threshold values of 0.7 mM for Ca^{2+} and 3 mM for Mg^{2+} . In other experiments (not shown) utilizing light scattering in which case very low vesicle concentrations can be used, the observed threshold values were found to increase somewhat with decreasing vesicle concentration. More importantly, subthreshold concentrations of Mg^{2+} significantly lower the concentrations of Ca^{2+} required for aggregation. Subthreshold concentrations of Mg^{2+} also dramatically reduce the threshold for the Ca^{2+} -induced permeability of PS vesicles (Figure 4B). This result is in accord with the apparent dependence of the Ca^{2+} -induced permeability increase on aggregation of the vesicles shown earlier (Figure 3).

The concentration difference between the effects of Ca^{2+} and Mg^{2+} , and the apparent synergism when both are present, appears to be related to the difference in the affinity of Ca^{2+} and Mg^{2+} to PS shown in Figure 1. Comparison of the binding curves for Mg^{2+} and Ca^{2+} (Figure 1) with the concentrations of Ca^{2+} and Mg^{2+} sufficient for aggregation (Figure 4A) indicates that rapid aggregation requires a large reduction of the surface charge density of the vesicles. This dependence is more clearly demonstrated in Figure 4C, where the aggregation results of Figure 4A are replotted vs. the extent of cation binding as calculated from the apparent binding constants obtained from Figure 1. Aggregation in the presence of both Ca^{2+} and Mg^{2+} also depends strictly on the extent of total cation binding, estimated by assuming competition between the cations. This assumption applies only to the initial cis complex since the unique (trans) Ca/PS complex must be formed following aggregation. Therefore the reduction of the threshold concentration of Ca^{2+} required for aggregation can be quantitatively accounted for by consideration of the reduction of surface charge density by Ca^{2+} and Mg^{2+} binding (cis complex). The reduction of the threshold concentration of Ca^{2+} required for release seems to be related to the ratio of bound Ca^{2+} per PS (Figure 1) and its mechanism is discussed below.

The experiments indicating that the presence of Mg^{2+} lowers the threshold Ca^{2+} concentrations required for release of CF (Figure 4B) were somewhat complicated by the release that occurs when Mg^{2+} alone aggregates the vesicles. At temperatures above 20 °C, Mg^{2+} -induced aggregation is accompanied by relatively little fusion. Therefore it was of great interest when we observed that the increase in CF permeability of sonicated PS vesicles induced by Mg^{2+} was only transient⁴ (30–60% of complete release) in contrast to that of Ca^{2+} . Details of the relationship between the transient Mg^{2+} -induced permeability increase and fusion will be presented separately (Portis et al., 1979). However, during the course of these experiments we observed that larger vesicles (obtained by

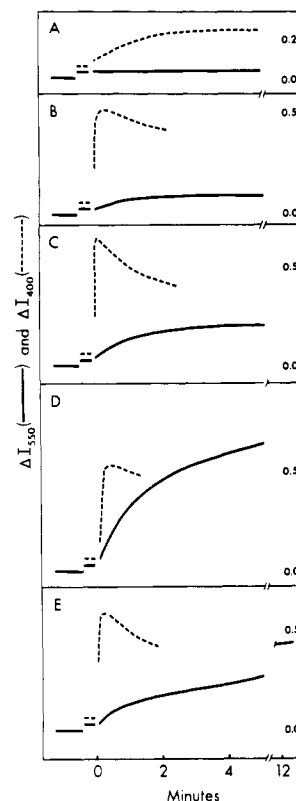


FIGURE 5: Time course of aggregation (---) and release (—) of CF from large PS vesicles (20 μM) after addition of Mg^{2+} and Mg^{2+} with Ca^{2+} . The decrease in light scattering observed in B–E is due to the formation of large visible aggregates: (A) 3 mM Mg^{2+} ; (B) 5 mM Mg^{2+} ; (C) 10 mM Mg^{2+} ; (D) 3 mM Mg^{2+} + 1 mM Ca^{2+} ; and (E) 5 mM Mg^{2+} + 0.5 mM Ca^{2+} . Temperature was 22 °C. Aggregation was measured by 90° light scattering (ΔI_{400}) and CF release by fluorescence intensity (ΔI_{550}).

differential centrifugation from the usual sonicated preparation; see Materials and Methods) were considerably more resistant to the Mg^{2+} -induced release of CF and fusion (as determined by an increase in vesicle diameter). For example, 10 mM Mg^{2+} induces the release of approximately 20% of the entrapped CF (Figure 5C) from the larger PS vesicles as compared with approximately 50–60% of the usual sonicated preparation, although aggregation of the vesicles was not markedly different (Portis et al., 1979). There is, therefore, a dependence on vesicle size for the CF release induced by Mg^{2+} , although the kinetics of CF release by Ca^{2+} alone were not affected by the size of the vesicles (data not shown). To minimize the effects of Mg^{2+} alone, we used the larger vesicles to investigate in more detail the effects of Mg^{2+} in combination with Ca^{2+} . The kinetics of light scattering (aggregation) and CF release induced by Mg^{2+} and Mg^{2+} with Ca^{2+} using these vesicles are shown in Figure 5. Near-threshold concentrations of Mg^{2+} (3 mM) and Ca^{2+} (1 mM) caused a very dramatic increase in CF permeability (Figure 5D) compared with Figure 3B. However, lower concentrations of Ca^{2+} (0.5 mM) with 5 mM Mg^{2+} (Figure 5E) or with 3 mM Mg^{2+} (not shown but similar to Figure 5E) were not as effective. In these cases, an initial fast release was followed by a slower but continuous release. In either case, the release is much higher than that obtained by Mg^{2+} alone (Figures 5A and 5B). Thus very low concentrations of Ca^{2+} are sufficient to cause a large increase in CF permeability in the presence of Mg^{2+} concentrations which in combination will aggregate the vesicles (Figures 5D and 5E). This result may be compared with the previous data showing that Ca^{2+} binding (trans complex) is not inhibited by high Mg^{2+} concentrations. The more dramatic effect of

⁴ More recent experiments on $^{22}\text{Na}^{+}$ permeability over longer time periods (hours) indicate that ^{22}Na permeability increase induced by Mg^{2+} is also transient.

near-threshold Mg^{2+} and Ca^{2+} concentrations (Figure 5D) is readily understood by the following consideration. The presence of Mg^{2+} will reduce somewhat the degree of Ca^{2+} binding (in a cis complex), but the further reduction of the surface charge density due to Mg^{2+} binding (Figure 1) dramatically enhances aggregation (see Figure 4A), which is kinetically rate limiting under these conditions (i.e., sub-threshold Ca^{2+}). At lower Ca^{2+} concentrations (0.5 mM) or higher Mg^{2+} concentrations (5 mM), the extent of Ca^{2+} binding (cis complex) is reduced to such an extent that a rapid increase in permeability (which is presumably related to the formation of the "trans" PS/Ca complex) does not occur in spite of aggregation.

The enhanced CF release observed at subthreshold concentrations of Ca^{2+} with Mg^{2+} and the binding of Ca^{2+} in the presence of high Mg^{2+} (Figure 1) are correlated with enhanced fusion of the PS vesicles and the formation of the characteristic PS/Ca complex. Formation of the cochleate lipid cylinders occurs when sonicated PS vesicles are dialyzed against buffer containing both Ca^{2+} and Mg^{2+} (0.05 mM Ca^{2+} + 5 mM Mg^{2+} or 0.1 mM Ca^{2+} + 3 mM Mg^{2+} for 3 h at 37 °C). This was established by freeze-fracture electron microscopy (Papahadjopoulos et al., 1977) in collaboration with Dr. W. J. Vail (Frostburg State College). X-ray diffraction analyses of these preparations show the presence of 4.1- and 4.5-Å diffraction lines indicating that the crystalline PS/Ca complex is formed (Table I). Differential scanning calorimetry shows a nearly complete disappearance of the low temperature transition after dialysis in buffer containing 0.1 mM Ca^{2+} and 5 mM Mg^{2+} , indicative of a shift of the T_c to high temperature. Under the same conditions, in the absence of Ca^{2+} , the PS transition is at ~20 °C.

The apparent synergism between Ca^{2+} and Mg^{2+} discussed above can be interpreted in terms of a reduction of electrostatic repulsion by Mg^{2+} leading to the formation of a "trans" PS/Ca complex. Following addition of Mg^{2+} alone (at concentrations at or above threshold) the reduced surface charge density leads to aggregation of intact PS vesicles with a layer of water of approximately 7 Å still separating the bilayers. A relatively small degree of fusion and release of vesicle contents is observed under these conditions, and the vesicles remain fluid at temperatures above 25 °C. When both Ca^{2+} and Mg^{2+} are present, each of the ions contributes in reducing the surface charge density and thus enhancing the frequency of close contacts. Such close contacts will then lead to the formation of the anhydrous "trans" PS/Ca complex at subthreshold concentrations of Ca^{2+} . Thus, the apparent synergism between Ca^{2+} and Mg^{2+} appears to be due to the balance between electrostatic repulsive forces, reduction of surface charge density (by either Ca^{2+} or Mg^{2+}), and formation of the anhydrous PS/Ca complex between adjacent vesicles. The same phenomena could also explain the apparent high cooperativity of the Ca^{2+} effects in the absence of Mg^{2+} (Papahadjopoulos et al., 1977) and the previously unexplained time-dependent shift of the threshold Ca^{2+} concentration for ^{22}Na permeability to lower values (Papahadjopoulos & Ohki, 1970).

Inhibitory Effects of Spectrin and the Relationship to the Requirement for Close Contact between Apposed Membranes. Spectrin has been reported recently to inhibit Mg^{2+} - and Ca^{2+} -induced fusion and the upward shift in the phase transition temperature of dimyristoylphosphatidylglycerol vesicles (Mommers et al., 1977). An explanation for this effect was not suggested. We investigated the influence of spectrin on the effects of Ca^{2+} on PS vesicles, anticipating that the effects of spectrin might possibly be explained by a steric

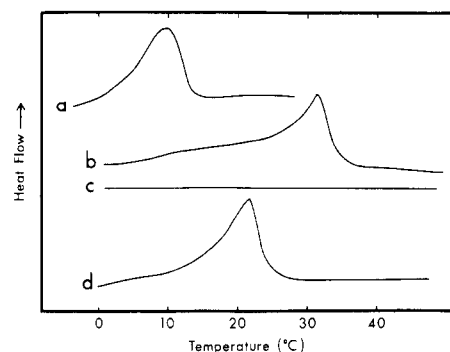


FIGURE 6: Differential scanning calorimeter tracings of the endothermic phase transition of PS vesicles. (a) PS multilamellar preparation in buffer ($\Delta H = 4.5 \pm 0.5$ kcal/mol). Preparations of sonicated PS vesicles showed an endothermic peak which is broadened and shifted to lower temperatures (midpoint is -3 °C) with relatively reduced ΔH (1.8 ± 0.5 kcal/mol). Such preparations have to be concentrated by ultrafiltration instead of centrifugation as in samples a-d. (b) Sonicated PS vesicles (2 $\mu\text{mol/mL}$) with spectrin (2:1 w/w) following dialysis at 22 °C for 3 h vs. buffer containing 1 mM CaCl_2 and collection by centrifugation; (c) same as b but without spectrin; and (d) same as b except dialysis vs. buffer containing 5 mM MgCl_2 ($\Delta H = 4.4 \pm 0.5$ kcal/mol).

interference with the requirement for close contact between the vesicles for formation of the trans-PS/Ca complex as discussed above. As shown in Figure 6, spectrin (1:2, w/w) inhibits the marked shift in the T_c of PS that normally occurs after Ca^{2+} addition (≥ 1 mM), inducing a new transition centered at 30 °C. This is similar to the limited upward shift of the T_c occurring at subthreshold Ca^{2+} concentrations (Jacobson & Papahadjopoulos, 1975). X-ray diffraction analysis of the sample at 22 °C revealed the presence of 4.2 Å spacing characteristic of hexagonal acyl-chain packing as contrasted to the highly ordered chain packing of the PS/Ca complex without spectrin. Freeze-fracture replicas of similarly prepared samples were devoid of cochleate lipid cylinders but otherwise showed particles of ill-defined morphology and less than 1000 Å in size. Therefore, the effects of Ca^{2+} on PS vesicles in the presence of spectrin resemble more closely those of Mg^{2+} or subthreshold Ca^{2+} concentrations. However, the protective effect of spectrin was observed to be time dependent, as the normal PS/Ca X-ray diffraction pattern and the disappearance of phase transition were found following continued incubation at 22–25 °C for 12 h. Incubation at 37 °C slowed down this conversion since, after 12 h, the sample still exhibited a detectable transition at 30 °C. In experiments similar to those above, the presence of spectrin did not affect the transition temperature of PS following Mg^{2+} addition (Figure 6).

Centrifugation experiments on sucrose density gradients demonstrated that spectrin binds to the vesicles in the presence of either Ca^{2+} or Mg^{2+} as indicated by an isopycnic sedimentation at a density greater than that of lipid with Ca^{2+} or Mg^{2+} alone. Sonicated PS vesicles prepared in standard NaCl buffer (2 μmol per mL) were incubated for 3 h at 22 °C with spectrin (0.7 mg/mL) in the presence or absence of Ca^{2+} (1 mM) and Mg^{2+} (3 mM). They were then centrifuged on step sucrose gradients as described in Materials and Methods. Following a 16-h centrifugation at 20 °C, PS vesicles with Ca^{2+} alone were located quantitatively at the interface between densities 1.11 and 1.13 g/mL. PS vesicles with Ca^{2+} and spectrin were located lower, at the interface between 1.13 and 1.15 g/mL. Similarly PS vesicles with Mg^{2+} alone settled between 1.01 and 1.11 g/mL, while PS vesicles with Mg^{2+} and spectrin settled between 1.11 and 1.13 g/mL. In the absence of lipid, more than 70% of the protein was found above the

Table II: Binding of Ca^{2+} and Mg^{2+} to PS in the Presence of Spectrin

sample ^a	molar ratios		
	Ca^{2+}/PS	Mg^{2+}/PS	$\text{Ca}^{2+}/\text{Mg}^{2+}$
PS vs. 4.5 mM Mg^{2+}		0.45	
PS + spectrin vs. 4.5 mM Mg^{2+}		0.46	
PS vs. 0.29 mM Ca^{2+}	0.25		
PS + spectrin vs. 0.29 mM Ca^{2+}	0.27		
PS vs. 4.5 mM Mg^{2+} and 0.29 mM Ca^{2+}	0.32	0.13	2.46
PS + spectrin vs. 4.5 mM Mg^{2+} and 0.29 mM Ca^{2+}	0.08	0.40	0.20

^a Sonicated PS vesicles containing the ionophore X-537 A were incubated with spectrin (2:1 lipid/protein (w/w)) at room temperature for 1 h and then dialyzed vs. buffers containing 100 mM NaCl and the indicated amount of divalent cations for 3 h at 37 °C. The samples were analyzed as described in Materials and Methods and the calculated ratios are shown. Duplicate determinations agreed within 5% of the given values.

layer of density 1.13 g/mL. The amount of spectrin bound to the lipid in the presence of Ca^{2+} was found to be 0.3–0.4 mg per mg of lipid and constituted 55% of the initial total protein added. Equilibrium dialysis experiments revealed that Ca^{2+} binding to PS (at 0.3–2 mM Ca^{2+} concentration) is not appreciably affected by spectrin binding and therefore cannot account for its effect (Table II).

Spectrin was also found to protect CF-containing vesicles from the rapid release of CF that normally follows Ca^{2+} addition (Figure 7). Maximal protection was found when spectrin was added at ratios of approximately 6 spectrin/vesicle and was observed even when high concentrations of Ca^{2+} (10 mM) were added. Significant effects were observed at ratios of 1 spectrin/vesicle. Albumin (human serum) added to PS vesicles in experiments similar to those in Figure 7 had no inhibitory effect even at ratios of protein to lipid 2:1 (w/w).

The protective effects of spectrin and their transient nature could be readily explained on the basis of spectrin binding to the surface of PS vesicles in the presence of Ca^{2+} . Such binding would be expected to result in steric interference with the close contact collisions between the bilayers of apposed vesicles following reduction of surface charge due to Ca^{2+} binding. This interference would inhibit the formation of the highly ordered intermembrane PS/Ca complex and thus inhibit the release of vesicle contents and fusion. Steric interference of this nature is apparently not limited to proteins as similar protective effects against Ca^{2+} -induced CF release have also been observed with PS vesicles containing a low mole percent of gangliosides or globosides which have large head groups (4–5 sugar residues) as compared with those containing equal amounts of cerebroside, which have only one sugar moiety (A. Portis, unpublished results).

The following experiment was designed in order to verify the above conclusion concerning the inhibitory effect of spectrin on the formation of the PS/Ca "trans" complex. The relative binding of Ca^{2+} and Mg^{2+} on PS vesicles in the presence of spectrin was determined. As shown in Table II, Ca^{2+} is not effective in competing with Mg^{2+} for PS binding in the presence of spectrin. For example, at concentrations of 0.29 and 4.5, respectively, there is 0.08 Ca^{2+}/PS and 0.40 Mg^{2+}/PS (Table II, line 6), while in the absence of spectrin the same ratios were 0.32 and 0.13 (line 5). Controls indicate that the binding of neither Ca^{2+} or Mg^{2+} alone is affected by spectrin. The ratios in the presence of spectrin are in close agreement with values calculated from the apparent binding constants. Therefore, we conclude that cation binding in the presence of spectrin is limited to the cis complex. These results provide

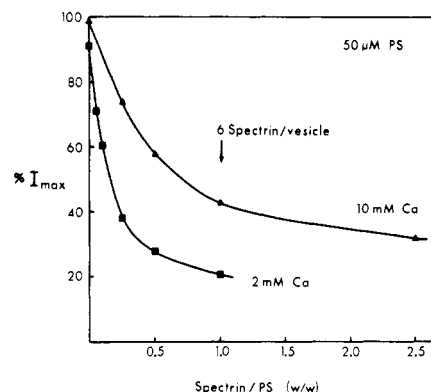


FIGURE 7: Spectrin inhibition of CF release induced by Ca^{2+} addition to sonicated PS vesicles. The increase in fluorescence of CF upon Ca^{2+} addition to the vesicles in the presence of spectrin was biphasic: an initial release dependent on the amount of spectrin present similar to that caused by Ca^{2+} alone followed by a much slower release. The extent of release following Ca^{2+} addition is shown after 2 min for 10 mM Ca^{2+} and 5 min for 2 mM Ca^{2+} since nearly complete release was obtained after these times in the absence of spectrin. The addition of spectrin alone had very little effect on CF release. The initial fluorescence of the vesicles before addition of Ca^{2+} was ~7% of maximal. Addition of subthreshold Ca^{2+} concentrations (0.5–1.0 mM) to the vesicles with spectrin did not offer increased protection against subsequent addition of higher Ca^{2+} concentrations. Spectrin and Ca^{2+} were added in small aliquots ($\leq 100 \mu\text{L}$) to vesicles (50 μM PS) in 2 mL of buffer. The temperature was 22 °C.

further substantiation to our conclusion that the presence of spectrin inhibits the formation of the intermembrane (trans) complex. They also provide further confirmation to our earlier conclusion that the unexpected effectiveness of Ca^{2+} in displacing Mg^{2+} from PS vesicles is related to the formation of such a trans complex.

General Discussion and Conclusions

Fusion of PS vesicles induced by Ca^{2+} has been demonstrated earlier by morphological and other studies (Papahadjopoulos et al., 1977). In this paper, we have followed the kinetics of the interaction between PS vesicles in the presence of Ca^{2+} and Mg^{2+} by following the increase in light scattering (which is related to both aggregation and fusion) and also by following the release of vesicle contents (which is an indirect assay for fusion indicating the loss of vesicle integrity). We have presented evidence for the occurrence of several specific complexes between divalent metal ions and the head groups of phosphatidylserine vesicles, and we have studied in some detail their possible relationship to the mechanism of membrane fusion. All the available evidence indicates that there are two different complexes formed by Ca^{2+} : One complex is formed when the cations bind only to individual vesicles and another complex is formed when the vesicles can come to close apposition. The latter complex, which has unique structural features, appears to be correlated with the initiation of membrane fusion.

The first type of complex occurs either at low Ca^{2+} concentrations (where residual electrostatic repulsive forces prevent vesicle aggregation) or in the presence of spectrin, even at high concentrations of Ca^{2+} . The characteristics of this complex are the following: (a) the phase transition is shifted to higher temperatures by only 22 °C (maximum); (b) the packing of the acyl chains below the phase transition is hexagonal (4.2 Å); (c) the integrity of the vesicles is maintained with only limited release of their contents and limited increase in size; (d) the formation of the complex does not involve a large release of heat. The complex formed by Mg^{2+} at both low and high concentrations shows similar charac-

teristics. We propose that this complex is formed only when the cations (Ca^{2+} or Mg^{2+}) bind to PS head groups on only one bilayer (cis complex).

The second type of complex is formed with Ca^{2+} only under conditions allowing the close apposition of the PS vesicles. This complex displays strikingly different characteristics: (a) the phase transition is increased to approximately 130 °C; (b) the packing of the acyl chains is highly ordered (4.1 and 4.5 Å) and the short lamellar spacing (53 Å) indicates direct apposition of the bilayers with no significant amount of free water in between; (c) the vesicles release their contents rapidly and fuse into larger structures; (d) the formation of the complex is accompanied by a large release of heat (5.5 kcal/mol). We propose that this complex involves a polydentate chelation of Ca^{2+} with the head groups of PS from apposed membranes in an anhydrous intermembrane arrangement (trans complex). Our use of the term "anhydrous" refers to the absence of free water and does not exclude the presence of a limited number of water molecules specifically associated with the complex. The nature and spatial organization of the complex will require further detailed structural studies. It appears that the formation of the trans PS/ Ca complex is a prerequisite step for the crystallization of the acyl chains and release of vesicle contents. Furthermore, we propose that this complex is of crucial importance for the observed fusion of the vesicles since it represents a transient destabilization of the closely apposed membranes.

Formation of the trans complex would be limited by the large repulsive forces associated with the high surface charge density of the vesicles, which prohibit close contact between PS vesicles suspended in 0.1 M NaCl, pH 7.4, and low concentrations of Ca (<1 mM). This is consistent with the finding that the trans complex is not observed unless a threshold concentration of Ca (≥ 1 mM) is present, sufficient to induce a large reduction of the surface charge density and aggregation of the vesicles.

Interactions of PS vesicles with Mg^{2+} (in the absence of Ca^{2+}), even at concentrations which allow aggregation, appear to involve only the formation of the cis complex, with a thin layer of water remaining between the apposed membranes. The relative inability of Mg^{2+} to induce formation of a trans complex may be related to the different preferred coordination numbers of Mg^{2+} and Ca^{2+} (Williams, 1976) and to the less favorable energetics of dehydration of Mg^{2+} generally observed in studies of divalent cation complexes involving various ligands (Nancollas, 1966). The nature of the head group also appears to be important as the effects of Ca^{2+} and Mg^{2+} on synthetic phosphatidylglycerols appear to be more similar in many respects (Van Dijck et al., 1975) than those found with bovine brain PS.

One of the most significant observations presented in this paper is the apparent synergism between Ca^{2+} and Mg^{2+} . We propose that this synergism is related to the reduction of the surface charge density induced by Mg^{2+} which would allow formation of the PS/ Ca complex even at very low Ca^{2+} concentrations due to the enhanced possibility for close contact between vesicles. Thus although Mg^{2+} is important for facilitating the close contact between vesicles, it is eventually displaced from the PS surface due to the formation of the trans complex which is specific for Ca^{2+} .

The significance of these conclusions for a possible mechanism for fusion of two stable phospholipid membranes can be obtained from a consideration of the minimal requirements for fusion: (1) close apposition; (2) destabilization; (3) formation of fused membrane with relief of destabilization.

It is obvious that close apposition of two membranes is an essential prerequisite for fusion. Recent studies indicate the existence of repulsive forces even between "neutral" phospholipid membranes (Le Neveu et al., 1976). It is therefore essential to establish conditions that promote membrane apposition to distances of only a few angstroms. However the observation that many types of vesicles can be aggregated by various means without extensive fusion (Papahadjopoulos, 1978) indicates that the close apposition of two bilayers does not necessarily result in fusion. Therefore destabilization must be an essential subsequent step for initiation of fusion. Specific proteins, lipids, and other compounds have been observed to enhance fusion but their mechanisms of action with regard to these minimal requirements are not completely clear at present. Our results indicate that Ca^{2+} may play a key role in membrane fusion by promoting both of the crucial steps mentioned above. Ca^{2+} can induce the close apposition of membranes (actually resulting in molecular contact with no intervening water) by interaction with the negatively charged phospholipids. Furthermore this interaction results in membrane destabilization since a phase transition (crystallization of the acyl chains) occurs with a release of heat in the region of contact. The heat release would enhance the rate of molecular mixing at the domain boundaries which were previously proposed as the initiation sites for fusion (Papahadjopoulos et al., 1977). The significance of trans-complex formation between Ca^{2+} and PS lies in the fact that the phase transition and release of heat occur only as the apposed membranes collapse into the anhydrous complex at close apposition. Similar considerations appear to apply to Ca^{2+} interactions with other acidic phospholipids (Papahadjopoulos et al., 1976; Van Dijck et al., 1975), but as mentioned previously differences with various acidic phospholipids in the extent of Mg^{2+} and Ca^{2+} specificity for trans-complex formation may exist and will require additional studies.

In considering whether Ca^{2+} -induced fusion of acidic phospholipid vesicles in model systems is relevant to in vivo fusion phenomena such as exocytosis, secretion, and synaptic vesicle fusion, the following points can be made. It is well established that the intracellular Ca^{2+} concentration is low, while that of Mg^{2+} is relatively high, and that Ca^{2+} is present at much higher concentrations extracellularly. There is now strong evidence that exocytotic and secretory events are triggered by an influx of Ca^{2+} into the cytoplasmic space (Poste & Allison, 1973; Rubin, 1974; Douglas, 1975). Furthermore, the available evidence suggests that the negatively charged phospholipids may be localized preferentially on the interior side of the plasma membrane of cells such as the erythrocytes (see review by Rothman & Lenard, 1977).

More extensive studies of phospholipid asymmetry are required, but, if this type of asymmetry is present also in other cells, then such an arrangement of Ca^{2+} and acidic phospholipid localization would ensure a forbidding asymmetry for the control of fusion as discussed previously (Papahadjopoulos, 1978). The intracellular Mg^{2+} could aid in promoting aggregation and close apposition of the secretory vesicles with the plasma membrane by reduction of the surface charge density of both membranes. The Mg^{2+} might thereby lower the threshold Ca^{2+} concentration required for fusion and perhaps also enhance the kinetics for subsequent events involving Ca^{2+} . The transient Ca^{2+} influx and increased concentration in the vicinity of the plasma membrane due to the appropriate stimulatory signal would allow Ca^{2+} access to the acidic phospholipids and induce fusion similar to that observed in model systems. Therefore it may be significant

that we have observed the formation of the crystalline PS/Ca complex and fusion in the presence of 0.05 mM Ca^{2+} and 5.0 mM Mg^{2+} which approach physiological concentrations. The question of whether the proposed mechanism can account for the extremely rapid kinetics of synaptic vesicle fusion and transmission (Heuser, 1977) will require the design of appropriate model systems.

The emphasis on Ca^{2+} and acidic phospholipids as the possible primary agents in inducing fusion does not exclude a role for proteins in *in vivo* fusion phenomena. Evidence against direct protein involvement in mammalian secretory cells has been suggested by the displacement of the intramembranous particles (IMPs) from the fusing areas in freeze-fracture electron microscope studies (Orci & Perrelet, 1978). This observation coincides well with the necessity of close contact for *Ca*²⁺-induced fusion and its inhibition by spectrin, with the exclusion of proteins from the rigid trans complex of Ca^{2+} and acidic phospholipid. However, fusion in other cases seems to be limited to particular regions of the membrane which are associated with IMP rosettes (Satir, 1974), linear IMP arrays (Heuser, 1977; Dreyer et al., 1973), or concentrations of IMPs (Venzin et al., 1977). These arrays have been proposed to consist in part of the Ca^{2+} gates (Venzin et al., 1977; Satir & Oberg, 1978), thereby localizing Ca^{2+} influx at the appropriate regions. Particular proteins also may be required for formation of lipid domains of high acidic phospholipid content in order to generate fusion-susceptible regions. PS/PC mixtures in model systems require a relatively high content of PS (~50–60%) in order to observe fusion, but much lower concentrations are sufficient if a partial phase separation of the two lipids occurs (Papahadjopoulos et al., 1978). Also, much lower concentrations are sufficient if phosphatidylethanolamine is present (Miller & Racker, 1976), but the reason for this effect is not yet clear. Present techniques have not clearly established whether lateral phase separations exist in natural membranes. However, there is evidence for the preferential interaction of certain proteins with various phospholipids and partial phase separations induced by proteins have been observed in some systems (Boggs et al., 1977a,b). The significance of these interactions with respect to fusion will require further studies.

Acknowledgments

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Expression of Multivalency in the Affinity Chromatography of Antibodies[†]

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Appendix: Derivation and Evaluation of Equations for Independent Bivalent Interacting Systems in Quantitative Affinity Chromatography

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ABSTRACT: The expression of multivalency in the interaction of antibody with immobilized antigen was evaluated by quantitative affinity chromatography. Zones of radioisotopically labeled bivalent immunoglobulin A monomer derived from the myeloma protein TEPC 15 were eluted from columns of phosphorylcholine–Sephacrose both in the absence and presence of competing soluble phosphorylcholine. At sufficient immobilized phosphorylcholine concentration, the variation of elution volume of bivalent monomer with soluble ligand was found to deviate from that observed for the univalent binding of the corresponding Fab fragment. In addition, the apparent

binding affinity of the bivalent monomer increased with immobilized antigen density. Use of equations relating the variation of elution volume with free ligand concentration for a bivalent binding protein allowed calculation of microscopic single-site binding parameters for the bivalent monomeric antibody to both immobilized and soluble phosphorylcholine. The chromatographic data not only demonstrate the effect of multivalency on apparent binding affinity but also offer a relatively simple means to measure microscopic dissociation constants for proteins participating in bivalent interactions with their ligands.

The enhancement of antibody–antigen binding affinity upon going from a monovalent antigen to a polyvalent one is now well documented (Karush, 1976). The term “intrinsic affinity” usually refers to the microscopic equilibrium association constant for the binding of a single antibody combining site to a single antigenic determinant. The affinity of a multivalent interaction is usually termed “avidity” or “functional affinity”. Several investigators have tried to evaluate the factor by which the intrinsic affinity will increase to give the functional affinity of a multivalent complex. Crothers & Metzger (1972) and Schumaker et al. (1973) developed theoretical equations in order to estimate this factor. Hornick & Karush (1972) and Gopalakrishnan & Karush (1974) approached the problem experimentally by comparing the association constants of anti-dinitrophenyl or anti-lactoside antibody obtained by equilibrium dialysis with monofunctional ligands to the constants obtained by antibody mediated neutralization of ϕ X 174 phage, to which these chemical groups have been multiply conjugated. These workers observed a dramatic increase in phage neutralization upon going from a monovalent Fab fragment through a bivalent IgG antibody to a decavalent IgM molecule. They did not emphasize, however, the crucial dependence of the enhancement factor on the density of the repeating antigenic determinants on the surface to which these groups were chemically attached. Another difficulty inherent in these studies was the comparison of data obtained by a binding technique, equilibrium dialysis, with those obtained by a phage neutralization technique, a biological phenomenon which is related to but not identical with primary binding.

We have approached the problem of assessing the quantitative expression of multivalency by using the recently developed method of quantitative affinity chromatography (Dunn & Chaiken, 1974, 1975; Chaiken & Taylor, 1976). This technique has been used to measure the binding affinity of proteins to their ligands by elution of zones of protein (in both the presence and absence of soluble ligand) on an insoluble matrix to which a ligand is covalently attached. Using this approach, we have measured the functional affinity of a bivalent antibody (TEPC 15) for its immobilized antigen (phosphorylcholine) as it varies with immobilized antigen density. We have compared the values for this functional affinity to the “intrinsic affinity” of a monovalent fragment derived from the same antibody. A mathematical expression was developed which can be used to calculate the microscopic intrinsic affinity of antibody–antigen systems from data obtained by quantitative affinity chromatography.

Experimental Section

Antibody and Derivatives. The phosphorylcholine binding IgA myeloma protein of the BALB/c plasmacytoma TEPC 15 was purchased from Litton Bionetics Inc., Kensington, MD. It was shown to be pure by polyacrylamide gel electrophoresis and immunoelectrophoresis against class specific antisera. Elimination of polymerized products was achieved by reduction and alkylation of the protein according to Miller & Metzger (1965). This procedure was also used to obtain radioactively labeled IgA monomer by using [14 C]iodoacetamide (57 mCi/mmol, Amersham, England) in the alkylation reaction. The resulting specific activity of the protein was 1400 dpm/ μ g.

IgA Fab fragments from TEPC 15 IgA monomer were prepared by papain digestion essentially according to Porter

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