J. Membrane Biol. 59, 115–125 (1981)

The Journal of
Membrane Biology

Calcium- and Magnesium-Induced Fusion of Mixed Phosphatidylserine/Phosphatidylcholine Vesicles: Effect of Ion Binding

Nejat Düzgüneş*, Shlomo Nir**, Jan Wilschut***, Joe Bentz, Carolyn Newton****, Archie Portis*****, and Demetrios Papahadjopoulos

Cancer Research Institute, University of California, San Francisco, California 94143, and
Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14263

Summary. The aggregation, leakage, and fusion of pure PS (phosphatidylserine) and mixed PS/PC (phosphatidylcholine) sonicated vesicles were studied by light scattering, the release of encapsulated carboxyfluorescein, and a new fusion assay which monitors the mixing of the internal compartments of fusing vesicles. On a time scale of 1 min the extent of fusion was considerably greater than leakage. The Ca^{2+} and Mg^{2+} concentrations required to induce fusion increased when the PS content of the vesicles was decreased, and/or when the NaCl concentration was increased.

Calculations employing a modified Gouy-Chapman equation and experimentally determined intrinsic binding constants of Na^+ and Ca^{2+} to PS were shown to predict correctly the amount of Ca^{2+} bound in mixed PS/PC vesicles. For vesicles composed of either pure PS or of mixtures with PC in 100 mM NaCl (4:1 and 2:1 PS/PC); the induction of fusion (on a time scale of minutes) occurred when the amount of Ca or Mg bound/PS molecule exceeded 0.35–0.39. The induction of fusion for both pure PS and PS/PC mixed vesicles (with PS exceeding 50%) can be explained by assuming that destabilization of these vesicles requires a critical binding ratio of divalent cations to PS.

Several mechanisms have been proposed for membrane fusion in phospholipid membrane systems. Evidence obtained with phosphatidylserine (PS)[1] membranes indicates strongly that the phase transition induced by Ca^{2+} and the resulting changes in lateral compressibility and structural defects may be involved in fusion (Papahadjopoulos et al., 1977). The shift in the endothermic transition of PS to much higher temperatures (Jacobson & Papahadjopoulos, 1975; Newton, Pangborn, Nir & Papahadjopoulos, 1978) is associated with the formation of an inter-membrane "trans" Ca^{2+}/PS complex which is essentially anhydrous (Papahadjopoulos, Portis & Pangborn, 1978; Portis, Newton, Pangborn & Papahadjopoulos, 1979). This complex may be the site of initiation of fusion (Papahadjopoulos et al., 1978; Portis et al., 1979).

The Ca^{2+}-induced aggregation and fusion of PS vesicles occurs at a threshold concentration of about 1 mM (Papahadjopoulos, Poste, Schaeffer & Vail, 1974); at this concentration Ca^{2+} is bound to the membrane surface at a ratio of 0.4 Ca^{2+} per phospholipid molecule (Portis et al., 1979; Newton et al., 1978). The rate of aggregation, release of aqueous contents, and fusion of PS vesicles increases dramatically above this threshold concentration (Düzgüneş & Ohki, 1977; Düzgüneş, 1978; Portis et al., 1979; Nir, Bentz & Portis, 1980*a*). Magnesium can also induce limited fusion of small unilamellar vesicles made of PS, but at a higher threshold concentration; this correlates with the fact that the intrinsic binding constant of Mg^{2+} to PS is lower than that of Ca^{2+} (Newton et al., 1978; Nir, Newton & Papahadjopoulos, 1978). The sequence of effectiveness of various divalent ions in inducing membrane aggregation and fusion also correlates well with their binding to PS (Ohki & Düzgüneş, 1979). The degree of divalent cation binding to the membrane surface may therefore be a significant factor in membrane fusion.

* *Mailing address for reprint requests:* Cancer Research Institute, University of California, San Francisco, California 94143.

** *Present address:* Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel.

*** *Present address:* Department of Physiological Chemistry, University of Groningen, 9712 KZ Groningen, The Netherlands.

**** *Present address:* Department of Biology, Kalamazoo College, Kalamazoo, Michigan 49007.

***** *Present address:* U.S. Department of Agriculture, Science and Education Administration, Agricultural Research and Department of Agronomy, University of Illinois, Urbana, Illinois 61801.

[1] The following abbreviations are used: PS, phosphatidylserine; PC, phosphatidylcholine; CF, carboxyfluorescein; TES, N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; DPA, dipicolinic acid.

0022-2631/81/0059-0115 $02.20
© 1981 Springer-Verlag New York Inc.

The studies on divalent ion binding to PS membranes (Newton et al., 1978; Nir et al., 1978) have also implicated the binding of Na^+, and this has been confirmed by NMR relaxation (Kurland, Newton, Nir & Papahadjopoulos, 1979*b*), ζ-potential measurements (Eisenberg, Gresalfi, Riccio & McLaughlin, 1979) and aggregation studies (Nir et al., 1980). Studies on the aggregation of PS vesicles and the release of entrapped carboxyfluorescin (CF) have indicated that a certain critical ratio of Ca^{2+} or Mg^{2+} bound per PS may be required for the destabilization of these vesicles (Nir et al., 1980), this destabilization being a prerequisite for fusion as suggested previously (Nir, 1977; Papahadjopoulos et al., 1977).

The critical divalent cation concentration which induces the aggregation of vesicles containing both phosphatidylcholine (PC) and PS is considerably higher than that of pure PS vesicles (Düzgüneş, 1978; Ohki & Düzgüneş, 1979). The extent of fusion of small unilamellar vesicles with multilamellar vesicles or with monolayer membranes is greatly reduced and the threshold Ca^{2+} concentration is increased when the membranes are made of a mixture of PS and PC (Papahadjopoulos et al., 1974; Ohki & Düzgüneş, 1979). Pure PC vesicles will not aggregate or fuse in the presence of 10 mM Ca^{2+} and 100 mM NaCl (Papahadjopoulos et al., 1974; Düzgüneş & Ohki, 1977). Increasing the PS fraction to 50% enables the vesicles to undergo fusion in this electrolyte, but at a very slow rate (Hoekstra, Yaron, Carmel & Scherphof, 1979; N. Düzgüneş, *unpublished observations*). When the phospholipid composition is in the range of pure PS to 2:1 PS/PC, Ca^{2+} or Mg^{2+} can induce rapid aggregation and fusion. This paper presents a detailed investigation of the fusion of PS/PC mixed vesicles in this higher PS range where the fusion rate is appreciable. Our aim is to elucidate the relationship between divalent cation binding to the membranes and their fusion.

We have studied the fusion of small ($\sim$250 Å) unilamellar vesicles made of pure PS or mixtures of PS and PC (>50% PS) by means of an assay which measures directly the rate and extent of membrane fusion; the assay monitors the fluorescent intensity of a reaction which takes place only in the interior aqueous space of fusing vesicles (Wilschut & Papahadjopoulos, 1979). We have compared the kinetics of fusion with the time of course of aggregation and release of internal aqueous contents of the vesicles. We have calculated the amount of Ca^{2+} or Mg^{2+} bound to PS in the mixed membranes and confirmed these results with direct binding measurements. We find that the threshold concentrations of divalent cations which induce fusion in these systems are closely related to the extent of their binding to the membrane.

Materials and Methods

Bovine brain phosphatidylserine and egg yolk phosphatidylcholine were purified as previously described (Papahadjopoulos & Miller, 1967; Papahadjopoulos et al., 1977) and stored under Argon at −50 °C in sealed ampules until use. $TbCl_3$ was obtained from Alfa (Danvers, Mass.), dipicolinic acid (DPA) from Sigma (St. Louis, Mo.) and carboxyfluorescein (CF) from Eastman Kodak (Rochester, N.Y.). The last was crystallized according to Blumenthal and coworkers (Blumenthal, Weinstein, Sharrow & Henkart, 1977). All other chemicals were of the highest purity commercially available. Water was twice distilled, the second time in an all-glass apparatus.

Small unilamellar vesicles were prepared by sonication as described by Papahadjopoulos, Nir and Ohki (1972). The lipids were mixed in chloroform, dried in a rotary evaporator and further in high vacuum for 30 min, suspended in the various aqueous media to be entrapped inside the vesicles, and sonicated for 1 hr in a bath-type sonicator under Argon. The temperature was maintained around 20 °C by circulating the water in the bath. The resulting clear suspension was then centrifuged at 25 °C for 1 hr at 115,000 × *g* to eliminate any large or multilamellar vesicles; the supernatant was collected for use in the experiments.

Fusion of the vesicles was monitored by means of the assay described previously (Wilschut & Papahadjopoulos, 1979), with some modifications. The vesicles were prepared in either of the following solutions: (*A*) 15 mM $TbCl_3$, 150 mM Na citrate, 2 mM Tris(hydroxymethyl)methyl-2-amino ethanesulphonic acid (TES), 2 mM L-histidine, pH 7.4, and (*B*) 150 mM Na Dipicolinate (DPA), 2 mM TES, 2 mM L-histidine, pH 7.4. The vesicles were separated from nonentrapped material by gel filtration on Sephadex G-75 (Pharmacia, Piscataway, N.J.) by elution with 100 mM NaCl, 2 mM L-histidine, 2 mM TES ("NaCl buffer") and 1 mM EDTA (pH 7.4). The EDTA was included to prevent Tb^{3+} binding to the vesicle surface during elution. Lipid concentrations were determined by a phosphate assay (Bartlett, 1959). For the fusion assay, the Tb-vesicles and the DPA-vesicles were mixed in a 1:1 molar ratio at a final lipid concentration of 50 μM in 1 ml of NaCl buffer containing 0.1 mM EDTA. Fluorescence was measured in an SLM 4000 fluorimeter (SLM Instruments, Champaign-Urbana, Ill.) by excitation at 276 nm; the emission was measured through a monochromator set at 545 nm (with the addition of a Corning 3-68 cutoff filter to eliminate the scattering contribution to the fluorescence). Full scale (100%) fluorescence was set by the following procedure: A portion of the Tb-vesicles were passed through a Sephadex G-75 column equilibrated with NaCl buffer to eliminate the external EDTA, which interferes with the reaction of Tb^{3+} and DPA. These vesicles were placed in 1 ml of NaCl buffer at a final concentration of 25 μM lipid, excess DPA (20 μM) was introduced, and the vesicle contents were released by addition of 1% (wt/vol) sodium cholate.

The fusion reaction was initiated by injecting an aliquot of a concentrated solution of the divalent ion (chloride salt) into the incubation mixture which was stirred constantly. The temperature was kept at 25 °C by circulating water around the cell. The output of the fluorimeter was recorded continuously on a strip chart recorder.

The release of the internal aqueous contents of the vesicles during fusion was measured in parallel experiments where 100 mM CF, 2 mM L-histidine, 2 mM TES, 0.1 mM EDTA (pH 7.4) was entrapped in the vesicles, separated from nonencapsulated material as above and CF release into the medium was measured by the increase in fluorescence intensity due to de-quenching of the dye fluorescence when diluted (Weinstein et al., 1977; Portis et al., 1979). Excitation was at 493 nm and emission above 520 nm (Corning 3-68 cutoff filter). 100% release was determined by adding 0.1% (wt/vol) Triton X-100 to the vesicle suspension; the fluorescence before detergent lysis (approx. 5%) was taken as 0% release.

Light scattering at 276 nm was measured to follow the aggregation of the vesicles simultaneously during the fusion assay by means of a second photomultiplier channel at 90° to the excitation beam. Here a Corning 7-54 bandpass filter was used to eliminate the contribution from fluorescence.

The binding of divalent cations to the vesicles was determined by equilibrium dialysis and atomic absorption spectroscopy as described in detail before (Newton et al., 1978; Portis et al., 1979). To determine the effect of sodium on Ca^{2+}-binding to phosphatidylserine, vesicles were prepared at a concentration of 10 μmol/ml in 100, 300, or 500 mM NaCl, buffered with 2 mM L-histidine, 2 mM TES, pH 7.4, and containing 0.1 mM EDTA. The Ca^{2+} ionophore A23187 was dissolved in the chloroform solution of the phospholipids before drying, at a molar ratio of 1:100 ionophore/lipid. After sonication and centrifugation the vesicles were dialyzed for 6 hr at 37 °C against the appropriate concentration of NaCl buffer without EDTA and containing 0.5 mM Ca^{2+}. Experiments performed at 25 °C gave identical binding results. After dialysis the contents of the dialysis bags were removed, sonicated for 5 min to insure homogeneity and aliquots were analyzed for phosphate and calcium. For the experiment with 1 M NaCl, vesicles were not prepared at this NaCl concentration, since they aggregated extensively, but rather at 100 mM NaCl and then dialyzed against the higher concentration. For the experiments with mixed PS/PC vesicles the samples were dialyzed against a bulk solution containing 2 mM Ca^{2+} in 100 mM NaCl buffer. The amount of Ca^{2+} was determined with a Perkin-Elmer 370 A atomic absorption spectrophotometer.

Theoretical Analysis of Binding

The theoretical procedure has been described in detail in connection with binding studies on pure PS vesicles (Nir et al., 1978; Bentz & Nir, 1980). Here we present only an outline with emphasis on some points. To simplify the treatment we will assume that the cations present initially in solution interact mainly with the negatively charged component in a mixture (Papahadjopoulos, 1968; Seimiya & Ohki, 1973). The comparison with experimental results will be a test for the applicability of this assumption. The concentration of a cation i of valency z at a distance x from the surface is given by

$$C_{iz}(x) = C_{iz} Y(x)^z \tag{1}$$

where C_{iz} is its solution concentration, far away from the surface, that is, the solution value outside the dialysis bag in a dialysis experiment. The term $Y(x)^z$ is the Boltzmann factor,

$$Y(x)^z = \exp(-ze\psi(x)/kT) \tag{2}$$

in which e is the charge of a proton, $\psi(x)$ is the electrostatic potential at a distance x from the surface, k is the Boltzmann constant and T is the absolute temperature. The concentration of a cation i of valency z at the surface is given by

$$C_{iz}(o) = C_{iz} Y(o) = C_{iz} \exp(-ze\psi(o)/kT) \tag{3}$$

in which $\psi(o)$ is the surface potential, which is negative in our case, so that Eq. (3) expresses the enhancement of concentration of cations at the surface.

We supplement the Gouy-Chapman equation by accounting for the effect of cation binding. The decrease of the surface charge density σ, relative to the value $\sigma_{initial}$ (which corresponds to the case of very low ion concentrations), is

$$\sigma = \frac{\sigma_{initial}}{1 + Y(o)^2 K_i C_{i++} + Y(o) K_j C_{j+}} \tag{4}$$

in which K_i, K_j are the corresponding intrinsic binding constants (McLaughlin, Szabo & Eisenman, 1971; Nir et al., 1978). The intrinsic binding constants of Ca^{2+}, Mg^{2+} and Na^+ to PS have been determined previously (Nir et al., 1978). In the case of Ca^{2+} and Mg^{2+} we have considered only stoichiometric binding, i.e. 1:2, which is justified because the saturation levels have not exceeded the binding ratios of 0.5 Ca/PS or Mg/PS even in the presence of large solution concentrations of these cations. A treatment of the more general case, i.e., allowing for 1:1 binding of divalent cation to phospholipids, has been presented (McLaughlin, Grathwohl & McLaughlin, 1978; Bentz & Nir, 1980; McLaughlin et al., 1981; J.A. Cohen, *personal communication*), but the determination of such binding constants may need extensive binding studies with very large concentrations of divalent cations. It may be noted that this information may be needed for situations where the surface concentration of the negative component, e.g., PS in PC, is very dilute so that most PS molecules are surrounded by PC molecules and can only participate in 1:1 binding. In this case Ca^{2+} binding to PC would have to be taken into consideration.

Table 1. Experimental and calculated data for Ca^{2+} binding to mixed PS/PC vesicles[a, b]

System	Experimental ratio Ca^{2+}/PS	Calculated total Ca^{2+}/PS	Caculated tightly bound Ca^{2+}
I. Pure PS in the presence of 0.46 mM Ca^{2+} and varying amounts of Na^+ given below			
100 mM Na^+	0.27	0.29	0.28
300 mM Na^+	0.14	0.13	0.13
500 mM Na^+	0.11	0.07	0.07
1000 mM Na^+	0.08	0.03	0.03
II. Pure PS in the presence of 0.1 mM Ca^{2+} and variable Na^+			
300 mM Na^+	0.02	0.03	0.03
100 mM Na^+	0.14	0.14	0.13
III. Mixed vesicles in the presence of 2 mM Ca^{2+} and 100 mM Na^+			
Pure PS	0.45	0.39	0.38
PS/PC 2:1	0.39	0.36	0.35
PS/PC 2:3	0.35	0.32	0.31
PS/PC 1:4	0.27	0.25	0.24

[a] The experiments are described in Materials and Methods. The calculations are described in the text and in more detail in Nir et al. (1978). The binding constant used for Na^+ is 0.8 M^{-1}. The binding constant used for Ca^{2+} is 35 M^{-1} for the lower Ca^{2+} concentrations in I and II and 75 M^{-1} in III. The area per molecule used is 70 $Å^2$.

[b] PS, phosphatidylserine; PC, phosphatidylcholine.

Table 2. Calculated amount of Ca bound per PS in mixed PS/PC membranes[a, b]

System composition (PS/PC)	Calculated tightly bound Ca/PS at various Ca^{2+} concentrations (mM) in solution						Na^+ bulk concentration[c] (mM)
	0.7	1	2	3	5	10	
1) Pure PS	0.32	0.35	0.38	0.39	0.41	0.43	100
4:1	0.30	0.33	0.36	0.38	0.40	0.42	100
2:1	0.28	0.32	0.35	0.37	0.39	0.41	100
3:2	0.27	0.31	0.34	0.36	0.38	0.40	100
1:1	0.25	0.29	0.33	0.35	0.37	0.39	100
2:3	0.23	0.26	0.31	0.33	0.35	0.38	100
1:4	0.15	0.19	0.24	0.26	0.30	0.34	100
2) Pure PS	0.11	0.16	0.21	0.25	0.29	0.34	500
4:1	0.10	0.14	0.20	0.23	0.27	0.33	500
2:1	0.09	0.12	0.18	0.22	0.26	0.32	500
3:2	0.08	0.12	0.17	0.21	0.25	0.31	500
1:1	0.07	0.11	0.16	0.19	0.24	0.30	500
2:3	0.06	0.09	0.14	0.18	0.22	0.28	500
1:4	0.04	0.06	0.10	0.13	0.18	0.25	500
3) Pure PS	0.44	0.44	0.44	0.44	0.45	0.45	5
4:1	0.43	0.43	0.44	0.44	0.44	0.44	5
2:1	0.42	0.43	0.44	0.44	0.44	0.44	5
3:2	0.41	0.41	0.42	0.42	0.43	0.43	5
1:1	0.40	0.40	0.41	0.42	0.43	0.43	5
1:2	0.38	0.39	0.40	0.40	0.41	0.42	5
1:4	0.32	0.33	0.35	0.36	0.37	0.38	5

[a] The calculations are described in Nir et al. (1978). The binding constant used for Ca^{2+} is 75 M^{-1}.

[b] PS, phosphatidylserine; PC, phosphatidylcholine.

[c] The calculations are made with the Na^+ concentration 4 mM higher than marked to account for the buffer. This correction is important only for the low Na^+ cases.

and may lead to charge reversal at lower Ca^{2+} concentrations than in the case of pure PS. It may also be noted that in such cases the validity of the Gouy-Chapman equation is not guaranteed, because effects of discrete charge may play a role.

In the analysis we take into account the fact that the total measured amount of adsorbed cation i consists of (a) an amount tightly or chemically bound and (b) an amount residing in the double layer region. The latter amount is given by

$$n_{si} = n_i \int_0^{x_0} \{\exp(-ez_i\psi(x)/kT - 1\}\, dx \qquad (5)$$

in which n_{si} is the excess number of cation species i per unit area, n_i is the number of cation species i per unit volume in solution, i.e., away from the surface, and x_o is the thickness of the double layer region (where there is excess of cations above the solution concentration). In concentrated solutions n_{si} hardly changes by taking x_o to be infinity or, say, 20 Å. The amount of tightly bound cation is determined by subtracting the double layer contribution (b) from the total measured amount of adsorbed cation.

The distinction between these two quantities (a and b) is made in Table 1, in which we present both calculated and measured values. In Table 2 we have supplemented these values of theoretical predictions with the amount of Ca bound per PS as a function of Ca^{2+} concentration, PS/PC ratio, and Na^+ concentration.

Results

Binding of Divalent Cations to Phospholipids

The binding of divalent cations to phosphatidylserine has been demonstrated using a variety of techniques (Abramson, Katzman & Gregor, 1964; Bangham & Papahadjopoulos, 1966; Blaustein, 1967; Hauser & Dawson, 1967; Seimiya & Ohki, 1973; Newton et al., 1978; Ohki & Sauve, 1978; Nir et al., 1978; Portis et al., 1979; Kurland, Hammoudah, Nir & Papahadjopoulos, 1979*a*). Several studies on the fusion of PS vesicles have implied that a critical amount of divalent cations must be bound before the vesicles aggregate, destabilize and fuse (Portis et al., 1979; Nir et al., 1980). In order to elucidate the role of binding on the fusion reaction we have determined the amount of Ca^{2+} and Mg^{2+} bound to vesicles of varying surface charge density and in the presence of different concentrations of Na^+. The surface charge density influences the surface potential, which determines the surface concentration of divalent ion. Since Na^+ also binds to PS (Nir et al., 1978; Nir & Bentz, 1978; Eisenberg et al., 1979; Kurland et al., 1979*b*; Nir et al., 1980), varying the Na^+ concentration would also be expected to affect divalent ion binding. The results of the binding measurements are given in Table 1. The molar ratio of bound Ca^{2+} per PS was reduced drastically when the Na^+ concentration in the medium was increased while the Ca^{2+} concentration was kept constant. Our theoretical calculations predicted this trend, as shown in the middle column in Table 1. The amount of Ca^{2+} bound to each PS molecule was also lowered as the molar ratio of PS to PC in the membrane was decreased. Again, the theoretical values obtained were in good agreement with the experimental results. The calculated values in Table 1 were obtained by assuming that the intrinsic binding constants previously obtained for pure PS are also appropriate for different Na^+ concentrations and different mixtures of PS and PC. The agreement between the experimentally determined Ca/PS (which includes both the Ca^{2+} bound and the Ca^{2+} present in the double layer region) and the theoretically calculated total Ca/PS is quite good. In these experiments the vesicles were prepared in media containing the indicated concentrations of Na^+ so that the inside and outside concentrations were identical. The equilibration of Ca^{2+} across the vesicle membrane was assured by including the Ca^{2+} ionophore A23187 during vesicle preparation. The transport activity of the ionophore was checked in a parallel experiment with the Ca^{2+}-sensitive dye

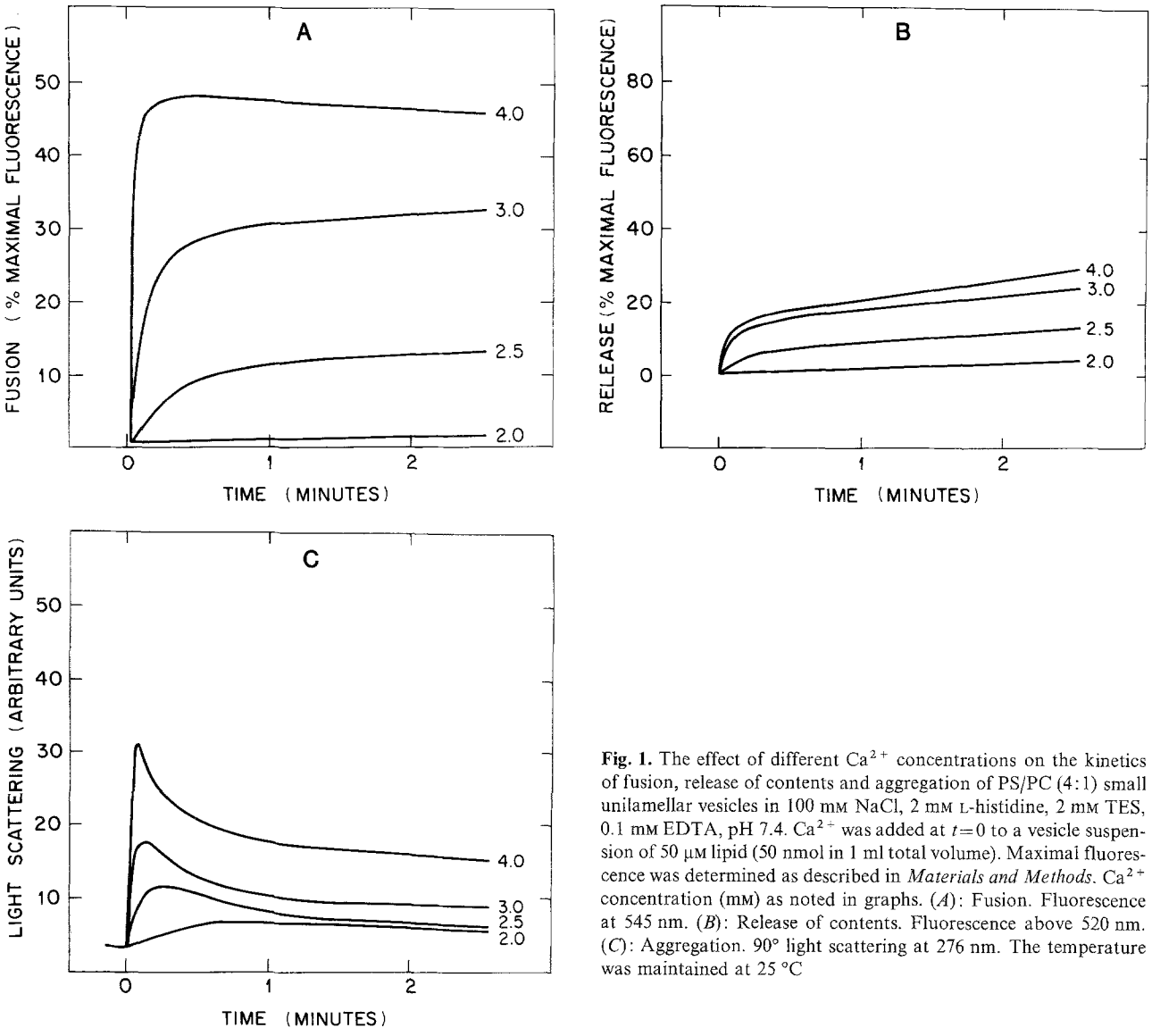

Fig. 1. The effect of different Ca^{2+} concentrations on the kinetics of fusion, release of contents and aggregation of PS/PC (4:1) small unilamellar vesicles in 100 mM NaCl, 2 mM L-histidine, 2 mM TES, 0.1 mM EDTA, pH 7.4. Ca^{2+} was added at $t=0$ to a vesicle suspension of 50 μM lipid (50 nmol in 1 ml total volume). Maximal fluorescence was determined as described in *Materials and Methods*. Ca^{2+} concentration (mM) as noted in graphs. (*A*): Fusion. Fluorescence at 545 nm. (*B*): Release of contents. Fluorescence above 520 nm. (*C*): Aggregation. 90° light scattering at 276 nm. The temperature was maintained at 25 °C

Arsenazo III[2]. The agreement between theory and experiment for the cases of mixed phospholipid vesicles (Table 1, Section *3*) implies that the dilution of the PS in the membrane does not significantly change the intrinsic binding constants. When the surface charge density is lower, the surface potential is reduced; hence the Boltzmann factor $Y(o)$ is diminished and so is the amount of Ca^{2+} near the surface. Neglecting the binding of Ca^{2+} to PC (McLaughlin et al., 1978) does not appear to influence significantly the results. Likewise, complications such as discrete charge effects are not significant, even in vesicles containing only 20% PS. While further experiments would be necessary to demonstrate this, its validity would imply that the binding of ions to biological membranes can be analyzed effectively by the relatively simple modified Gouy-Chapman theory.

The results of the theoretical analysis of binding of Ca^{2+} to pure PS and mixed PS/PC vesicles in the presence of different concentrations of Na^+ (5, 100, and 500 mM) are presented in Table 2. It is clear for all the mixtures used that the amount of bound Ca^{2+} was decreased at 500 mM Na^+ and increased at 5 mM Na^+. These results will be discussed in relation to membrane aggregation and fusion in the following section.

Aggregation, Fusion, and Release of Contents

The aggregation and fusion of small unilamellar vesicles made of PS/PC mixtures of varying molar ratios

[2] The influx of Ca^{2+} was followed by the change in the absorbance spectrum of Arsenazo III entrapped in PC vesicles after addition of Ca^{2+} to the external medium.

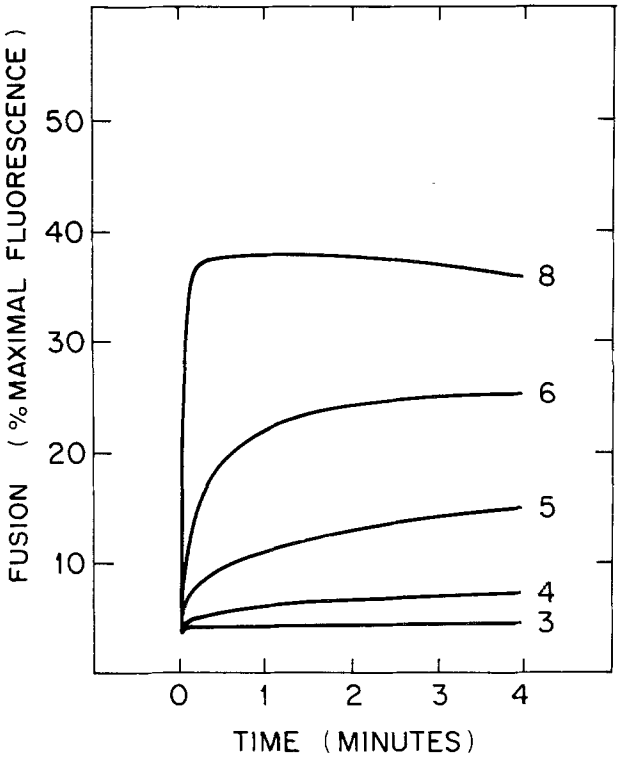

Fig. 2. The time course of the fusion of vesicles composed of PS/PC 2:1 in 100 mM NaCl buffer induced by various concentrations of Ca^{2+} (mM). Other conditions were as in Fig. 1

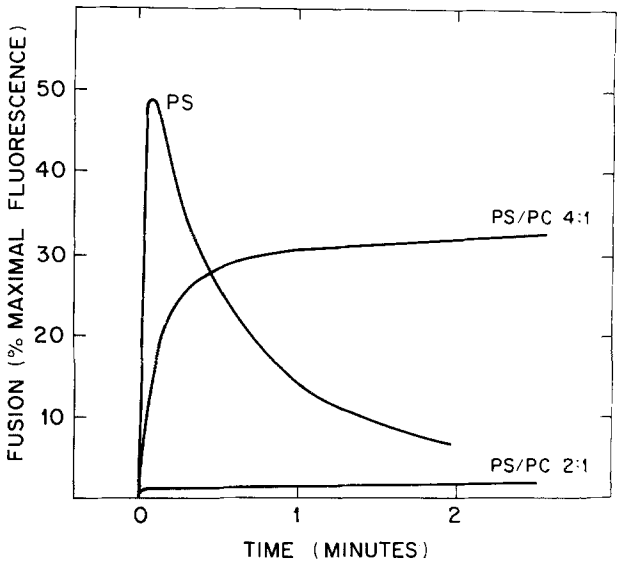

Fig. 3. The effect of membrane composition on the kinetics of vesicle fusion induced by 3 mM Ca^{2+} in 100 mM NaCl buffer. Other conditions were as described in Fig. 1

were monitored simultaneously by light scattering at 276 nm and by the fusion assay described previously (Wilschut & Papahadjopoulos, 1979). The assay measures the fluorescence intensity of the reaction between Tb^{3+} and DPA, which are encapsulated in different vesicle preparations and which can form the fluorescent $Tb(DPA)_3^{-3}$ complex upon intermixing of the internal aqueous contents following fusion. Outside the vesicles the reaction is inhibited by the presence of EDTA (0.1 mM) and Ca^{2+}. The details of the fusion assay, including studies on the order of the fusion reaction, the termination of the reaction by EDTA, and the mass action kinetics of fusion, have been described elsewhere (Nir, Bentz & Wilschut, 1980*b*; Wilschut, Düzgüneş, Fraley & Papahadjopoulos, 1980).

Figure 1*A* shows the time course of the fusion reaction induced by various concentrations of Ca^{2+} with vesicles composed of PS/PC (4:1). Appreciable fusion occurred only at a critical concentration of Ca^{2+}, and the higher the concentration of Ca^{2+}, the higher the initial rate of fusion. Previous studies on the fusion of pure PS vesicles have utilized the leakage of aqueous contents and light-scattering changes during the fusion reaction (Portis et al., 1979; Nir et al., 1980*a*). The kinetics of these processes are shown in Fig. 1*B* and *C* for the same vesicles as a comparison with the kinetics of fusion. Fusion was always accompanied by some release of entrapped CF, the extent of which increased with Ca^{2+} concentration. The same trend was observed for the extent of fusion. The initial rates of fusion, increase in light scattering, and release of contents also increased with the Ca^{2+} concentration. These findings support the conclusions reached earlier that the release of contents and increase in light scattering of PS vesicles are associated with fusion (Portis et al., 1979; Nir et al., 1980*b*). The new fluorescence assay also demonstrates that the degree of fusion initially exceeds that of leakage in these vesicles (Wilschut & Papahadjopoulos, 1979). The initial increase in light scattering indicates aggregation and fusion of vesicles; in both cases the light scatterer is a larger particle. However, as the aggregation and fusion proceeds, the intensity of scattered light reaches a maximum and then decreases slightly (Fig. 1*C*). This could be due to the formation of large clumps of vesicles and/or an interference effect which is expected when the size of the scattering particle approaches half the wavelength. Thus, for small aggregates (2–3 vesicles) the interference effect is not important, but when the aggregate size increases, especially at high Ca^{2+} concentrations, it has a substantial effect. This effect has been described in an analysis of 90° light scattering from fused PS vesicles (after addition of EDTA) in relation to the kinetics of fusion (Nir et al., 1980*b*). The pattern of the Ca^{2+}-induced light scattering increase in these vesicles (Fig. 1*C*) is different from the drastic increase observed for PS vesicles (Portis et al., 1979; Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980),

Table 3. Calculated amount of Mg bound per PS in mixed PS/PC membranes[a, b]

System	Calculated tightly bound Mg/PS at various Mg^{2+} concentrations in solution						
	1	5	10	20	25	30	50
Pure PS	0.27	0.35	0.38	0.40	0.40	0.41	0.42
PS/PC (2:1)	0.23	0.32	0.35	0.37	0.38	0.39	0.40

[a] In view of the new binding results of Portis et al. (1979) a binding constant of 20 M^{-1} is used for Mg^{2+}. Nir et al. (1980*a*) used a value of 10 M^{-1}. This smaller binding constant would reduce the amount of Mg bound by less than 0.05 in all cases.
[b] PS, phosphatidylserine; PC, phosphatidylcholine.

which is probably due to a combination of increase in size and the formation of an anhydrous *trans* Ca/PS complex with a different refractive index (Wilschut et al., 1980). The PS/PC vesicles undergo fusion to a limited extent, retain most of their contents (Fig. 1 *A* and *B*), and hence do not form collapsed structures. The light scattering increase is therefore limited.

When the mole fraction of PS in the vesicle membrane was decreased the Ca^{2+} concentrations required to induce fusion increased (Fig. 2). For vesicles made of 2:1 PS/PC the threshold was about 5 mM, whereas for the 4:1 vesicles it was 2.5 mM. The effect of membrane composition, and hence the surface charge density, is demonstrated clearly in Fig. 3, where the time course of fusion induced by 3 mM Ca^{2+} in vesicles composed of pure PS or mixed PS/PC is shown. The eventual decrease in the fluorescence seen in Figs. 1 *A*, 2 and 3 is caused by the leakage of vesicle contents into the medium and the entry of Ca^{2+} into the vesicles, both processes inhibiting the Tb/DPA reaction. The more drastic drop in Tb fluorescence attained with the PS vesicles compared to PS/PC, 4/1 (Fig. 3) is related to a larger release of contents (Wilschut et al., 1980). When the percentage of PS was reduced from 80 to 67% virtually no fusion occurred. It is apparent from the Ca^{2+} concentration dependence of the rate and extent of fusion of the mixed vesicles (Figs. 1 *A* and 2) and from the binding data given in Table 2, that fusion and binding are closely related. In fact, the threshold Ca^{2+} concentrations for each membrane composition corresponds to a bound Ca/PS ratio of about 0.39 in 100 mM NaCl (Table 2, Section 1). It should be noted here that this correlation does not apply to mixtures containing $\leqq 50\%$ PS in PC. In such cases, fusion is very slow or not observable at all, even at concentrations of Ca^{2+} high enough to give the expected ratio of bound Ca/PS (Table 2). A possible explanation for this phenomenon will be discussed later.

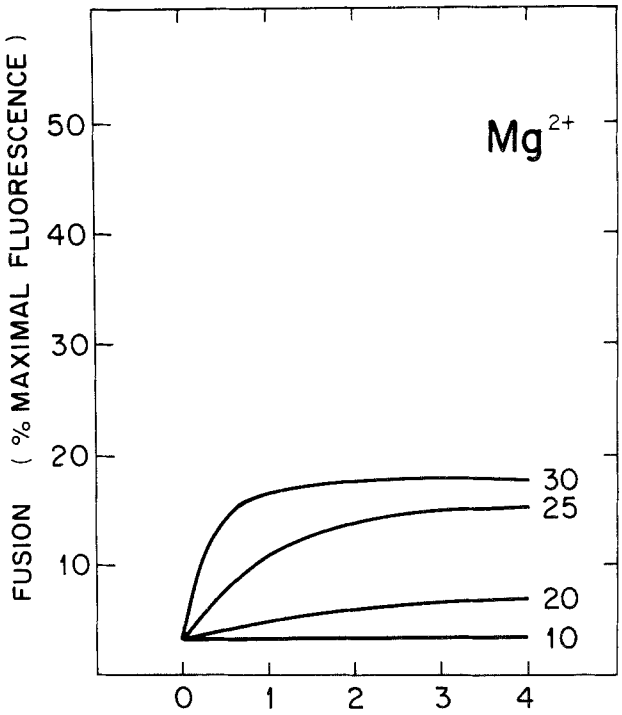

Fig. 4. The time course of the fusion of vesicles composed of PS/PC (2:1) in 100 mM NaCl buffer induced by various concentrations of Mg^{2+} (mM)

Magnesium has a lower intrinsic binding constant to PS than Ca^{2+} (Newton et al., 1978; Nir et al., 1978). Table 3 shows that higher concentrations of Mg^{2+} would be necessary to achieve the bound divalent cation/PS ratios that are associated with Ca^{2+}-induced fusion. Indeed, as shown in Fig. 4, 25 mM Mg^{2+} was required to attain the same extent of fusion as with 5 mM Ca^{2+} for 2:1 PS/PC vesicles within 1–2 min of the addition of the divalent ion. The initial rates of fusion were also slower for Mg^{2+} than for Ca^{2+}.

As discussed in the section on binding results, Na^+ has a profound effect on the binding of Ca^{2+} to PS. It would therefore be expected that the Ca^{2+}-induced fusion of phospholipid vesicles containing PS would be inhibited in the presence of high concentrations of Na^+ since the amount of bound Ca^{2+} per PS is lower. This is indeed the case, as shown in Fig. 5. Calcium at a concentration of 3 mM was unable to induce fusion in 4:1 PS/PC vesicles when the Na^+ concentration was raised to 500 mM, even though at 100 mM Na^+ it was able to induce considerable fusion (30% of maximal fluorescence) within 2 min. Similarly, when the Na^+ concentration was lowered to 5 mM the initial rate and the extent of fusion was increased. It is clear from Table 2 (Section 3) that the amount of Ca^{2+} bound drastically increases when the Na^+ concentration is lowered to 5 mM.

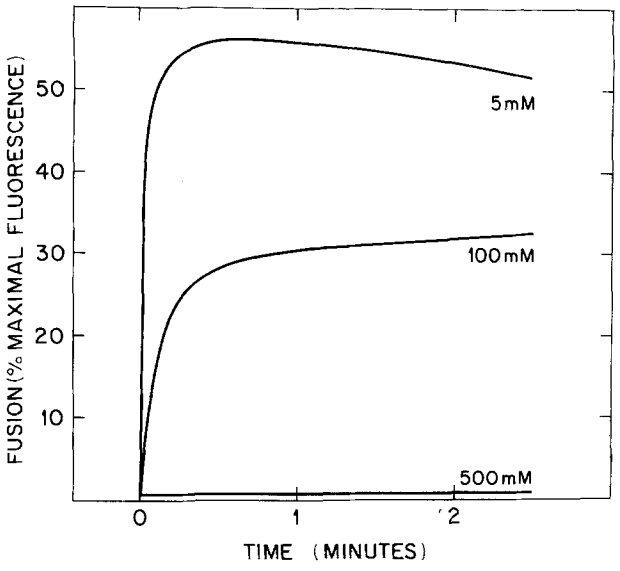

Fig. 5. The effect of the Na^+ concentration (mM) on the kinetics of fusion induced by 3 mM Ca^{2+} with vesicles composed of PS/PC 4:1. Other details are given in the legend to Fig. 1

It appears, therefore, that there is a close correlation between the amount of Ca^{2+} or Mg^{2+} bound to small unilamellar phospholipid vesicles of high PS content and the induction of fusion. It should be noted here that a critical ratio of divalent ion bound per PS is a necessary but not sufficient condition for fusion. For example, Ca^{2+} binding to mixtures of low PS percentage in PC or binding of Mg^{2+} to large unilamellar vesicles of pure PS (or PS/PC) does not result in fusion (Düzgüneş, Wilschut, Fraley & Papahadjopoulos, 1981; Wilschut et al., 1980; Wilschutz, Düzgünes & Papahadjopoulos, 1981).

Discussion

The primary aim of this study has been to show that when Ca^{2+} or Mg^{2+} are able to induce fusion of sonicated vesicles made of a particular PS/PC mixture, the amount of divalent cation bound to the PS component of the membrane exceeds a threshold ratio. To accomplish this aim, it was essential to distinguish between the stages of the fusion process (Nir, 1977; Papahadjopoulos et al., 1977): the close approach of vesicles, the destabilization of the bilayers, and the intermixing of the membranes and the internal contents of vesicles, It is known that the close approach or aggregation of pure PS vesicles is predictably determined by the total amount of cations bound to the vesicles and the ionic strength of the solution (Nir & Bentz, 1978; Nir et al., 1980*a*). Hence aggregation of these vesicles can be induced by Na^+ alone (Nir et al., 1980*a*; Day et al., 1980); however, there is no significant bilayer destabilization in this case, i.e., no leakage or fusion. The destabilization can be produced by the presence of Ca^{2+} or Mg^{2+} when their concentrations in solution are sufficient to induce the binding of a suprathreshold amount of divalent cation to the bilayers (Portis et al., 1979; Nir et al., 1980*a*).

The quantitative hypothesis that leakage is predictable from the amount of Ca bound to the vesicles was tested on pure PS vesicles (Nir et al., 1980*a*). To extend this analysis to mixed vesicle systems required showing that the binding of cations to these vesicles could be predicted from the modified Gouy-Chapman theory as outlined in the theoretical section of this work (*see also* Nir & Bentz, 1978, and Bentz & Nir, 1980, for more details). Table 1 shows that this theory reproduces the binding data for Ca^{2+} as the Na^+ concentration and the PS/PC ratio is varied. Hence we may be confident that in the systems studied the amount of Ca bound per PS molecule is fairly well known, within experimental error.

It must be noted that our calculations in Tables 1 to 3 do not consider the possibility of asymmetry of membrane composition across the bilayer. It has been suggested that in sonicated small unilamellar vesicles some asymmetry of membrane composition may exist because of the difference in packing density between the inner and outer monolayers (Bergelson & Barsukov, 1977). In a 1:1.8 mixture of PS/PC at neutral pH there is a slight preference of phosphatidylserine for the inner monolayer. Whereas the outside/inside ratio for total phospholipids is 2.3, the ratio for phosphatidylserine is 2.06 (Berden, Barker & Radda, 1975). This difference would alter the actual binding ratios, but not appreciably, as can be estimated from the values provided in Tables 2 and 3.

Figure 1*A–C* shows that the smallest concentration of Ca^{2+} needed to induce fusion is about 2 mM for 4:1 PS/PC vesicles in 100 mM Na^+, which corresponds to 0.36 Ca bound per PS molecule (*cf.* Table 2). However, more extensive fusion and leakage of CF occurs only after the Ca^{2+} concentration is increased to 2.5 mM, which corresponds to approx. 0.38 Ca bound per PS. For vesicles composed of 2:1 PS/PC mixtures the leakage threshold is between 4 and 5 mM Ca^{2+}, which corresponds to 0.38 Ca bound per PS (data not shown; however, *see* Fig. 2, since the fusion and leakage thresholds are identical as shown in Fig. 1*A* and *B*). Hence, the onset of bilayer destabilization and leakage is a function of the Ca bound to the PS in the bilayer. We do not mean to imply that these calculated values of the amount of Ca^{2+} bound are significant in absolute terms, since the data used to obtain binding constants are not yet sufficiently accurate; however, it is evident that the amount of Ca bound (and not merely the

concentration of Ca^{2+} in solution) is the relevant parameter for leakage and fusion.

The second aim of this study has been to show that pure PS and mixed PS/PC vesicles undergo substantial fusion (as defined by the intermixing of vesicle contents). The assay of Wilschut and Papahadjopoulos (1979) showed that in pure PS vesicles substantially more fusion occurs than leakage during the first minute after the addition of Ca^{2+}. The present study on mixed PS/PC vesicles also demonstrates that bilayer destabilization induces both leakage and fusion (hence the Ca bound is equally correlated to the fusion) and that following the addition of suprathreshold Ca^{2+} concentrations there is substantially more fusion than leakage during the early stages (*see* Fig. 1 *A–C*). The Ca^{2+}-induced fusion product of PS/PC vesicles is considerably different from that of pure PS vesicles. The latter release all their contents (Portis et al., 1979) and eventually form large cochleate structures with no aqueous space between the membranes (Papahadjopoulos, Vail, Jacobson & Poste, 1975). The mixed vesicles, on the other hand, retain most of their contents after undergoing fusion (Fig. 1 *B*). This difference is also apparent from the kinetics of the fusion reactions shown in Fig. 3. The fluorescence intensity of the Tb/DPA reaction eventually drops in the case of the PS vesicles because the contents are released into the medium where the fluorescence is quenched by EDTA and Ca^{2+}; this conclusion is supported by the observation of the extensive release of CF under the same conditions (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980). The PS/PC (4:1) vesicles do not collapse after fusion and the Tb/DPA complex is sequestered from the external medium inside intact vesicles. The reorganization of the membrane during fusion does, however, lead to the release of about 20% of the vesicle contents. It appears, therefore, that PC has a stabilizing effect and prevents the collapse of the internal aqueous space of the fused vesicles, at least within the timescale of our experiments.

The third aim of this study has been to compare the fusion induced by Mg^{2+} to that of Ca^{2+} and the effect of Na^+ concentration on fusion. If the predominant factor for leakage and fusion is the amount of divalent cation bound to the bilayer, then it would be expected that a substantially higher concentration of Mg^{2+} than Ca^{2+} would be needed to induce fusion in otherwise identical vesicle systems, since Mg^{2+} binds more weakly than Ca^{2+} (Newton et al., 1978; Nir et al., 1978; Portis et al., 1979). Figure 4 shows that this is indeed the case, where the threshold for 2:1 PS/PC vesicles in 100 mM Na^+ is at least 10 mM Mg^{2+}. It is of interest that this threshold corresponds to 0.35 Mg bound per PS; likewise in pure PS vesicles the Mg^{2+} threshold is ~6 mM (Wilschut et al., 1981) which corresponds to the same amount of Mg^{2+} bound per PS (Table 3). Hence, there is evidence for the hypothesis that fusion can be induced in these small unilamellar PS/PC vesicle systems whenever the divalent cation bound per PS exceeds a critical ratio of about 0.35–0.39, regardless of whether the cation is Ca^{2+} or Mg^{2+}.

We examined this hypothesis in more detail by altering the Na^+ concentration in the system. Since Na^+ competes with both Ca^{2+} and Mg^{2+} for binding to PS it is expected that lowering the Na^+ concentration would lower the divalent cation concentration needed to induce fusion and *vice versa*. Figure 5 illustrates that this is indeed the case. For 4:1 PS/PC vesicles in 3 mM Ca^{2+}, the fusion is completely inhibited with 500 mM Na^+, where the Ca^{2+} bound/PS is lowered to 0.25. Likewise, in 5 mM Na^+ fusion is greatly enhanced, and the Ca^{2+} bound per PS is increased to 0.44. It is of interest that for these extremes in Na^+ concentration the calculated thresholds of Ca bound per PS appear slightly shifted, such that for the induction of fusion less Ca^{2+} bound is needed when Na^+ is high and more Ca^{2+} bound is needed when Na^+ is low. However, quantification of these shifts requires further detailed binding analysis since, as shown in Table 1, the amount of Ca bound at high Na^+ is larger than predicted. In addition, we note that the process of vesicle-vesicle fusion consists of two stages and that increased Na^+ concentrations promote the process of close approach, while they inhibit the destabilization of vesicles induced by divalent cations. Thus the rate of fusion depends on the combined rates of the two processes, i.e., the close approach of vesicles and also their destabilization while in close promixity. At the vesicle concentration used in this study, the rate limiting step in fusion is vesicle aggregation (Wilschut et al., 1980; Nir et al., 1980*b*) whose rate depends on the Ca^{2+} concentration (Düzgüneş, 1978; Portis et al., 1979). Therefore, even above a threshold Ca bound/PS, the rate of fusion will depend on the rate of aggregation and hence on the concentration of Ca^{2+}, as shown in Figs. 1 *A* and 2. The same argument holds for Mg^{2+}-induced fusion, although in this case the rate of fusion is considerably slower than the aggregation (Wilschut et al., 1981).

Clearly the amount of divalent cation bound to PS is not the only parameter which must be considered in the analysis of vesicle fusion. The effects of temperature are well known (Kantor & Prestegard, 1975; Breisblatt & Ohki, 1975; Martin & MacDonald, 1976; Papahadjopoulos, Vail, Pangborn & Poste, 1976; Papahadjopoulos et al., 1977; Sun, Day & Ho, 1978; Hammoudah et al., 1979). Osmotic gradients across

the bilayer (Miller, Arvan, Telford & Racker, 1976) and the size of the vesicles (Wilschut et al., 1981) will affect the susceptibility to and the extent of fusion. Likewise, substituting phosphatidylethanolamine for PC as the neutral component in the vesicles will alter drastically the kinetics of the fusion process (Düzgüneş, Hong & Papahadjopoulos, 1980; Düzgüneş et al., 1981). All of these other parameters may represent different or additional mechanisms for the destabilization or stabilization of the vesicle bilayers. Above the phase transition temperature of the lipids, a critical parameter for inducing the fusion of sonicated PS and mixed PS/PC (PS > 50%) vesicles is the amount of Mg or Ca bound per PS. Relating this fact to the molecular structure of the vesicle bilayer will require substantially more knowledge about the physical chemistry of cation binding to vesicles (e.g., Kurland et al., 1979*b*; Gresh, 1980). The threshold ratio of divalent cation bound/PS probably indicates the formation of a particular coordination complex between neighboring PS molecules (Papahadjopoulos, 1968; Gresh, 1980). This complex may lead to local phase separation of the PS and PC (Ohnishi & Ito, 1974; Papahadjopoulos et al., 1974; Jacobson & Papahadjopoulos, 1975). The absence of detectable aggregation and fusion in vesicles composed of a larger PC fraction (PC > 50%; Papahadjopoulos et al., 1974; Düzgüneş & Ohki, 1977; N. Düzgüneş, *unpublished data*) may be a result of an inability of the divalent cation/PS complexes to cluster into sufficiently large domains to enable aggregation, destabilization and fusion. The exact relationship between the stoichiometry of ion binding, the size of PS domains, and fusion needs further clarification. Finally, it is of considerable interest to elucidate the relationship between the close approach of vesicles and bilayer destabilization which are jointly induced by cation binding.

This work was supported by Grants GM-26359 (D.P.), GM-23850 and CA-17609 (S.N.), a Fellowship CA-06190 (N.D.) from the National Institutes of Health, and by a Fellowship Grant (J.W.) from the Netherlands Organization for the Advancement of Pure Research (ZWO). The allotment of time by RPMI and SUNY at Buffalo Computer Centers is acknowledged. We thank Dr. F. Pecker (INSERM, Creteil, France) for assistance in some of the experiments, and H. Guillemin for the preparation of the manuscript.

References

Abramson, M.B., Katzman, R., Gregor, H.P. 1964. Aqueous dispersions of phosphatidylserine: Ionic properties. *J. Biol. Chem.* **239**:70

Bangham, A.D., Papahadjopoulos, D. 1966. Biophysical properties of phospholipids. I. Interaction of phosphatidylserine monolayers with metal ions. *Biochim. Biophys. Acta* **126**:181

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466

Bentz, J., Nir, S. 1980. Cation binding to membranes: Competition between mono-, di- and trivalent cations. *Bull. Math. Biol.* **42**:191

Berden, J.A., Barker, R.W., Radda, G.K. 1975. NMR studies on phospholipid bilayers. Some factors affecting lipid distribution. *Biochim. Biophys. Acta* **375**:186

Bergelson, L.D., Barsukov, L.I. 1977. Topological asymmetry of phospholipids in membranes. *Science* **197**:224

Blaustein, M.P. 1967. Phospholipids as ion exchangers: Implications for a possible role in biological membrane excitability and anesthesia. *Biochim. Biophys. Acta* **135**:653

Blumenthal, R., Weinstein, J.N., Sharrow, S.O., Henkart, P. 1977. Liposome-lymphocyte interaction: Saturable sites for transfer and intracellular release of liposome contents. *Proc. Nat. Acad. Sci. USA* **74**:5603

Breisblatt, W., Ohki, S. 1975. Fusion in phospholipid spherical membranes. I. Effect of temperature and lysolecithin. *J. Membrane Biol.* **23**:385

Day, E.P., Kwok, A.Y.W., Hark, S.K., Ho, J.T., Vail, W.J., Bentz, J., Nir, S. 1980. Sodium induced reversible aggregation of sonicated phosphatidylserine vesicles. *Proc. Nat. Acad. Sci. USA* **77**:4026

Düzgüneş, N. 1978. Divalent cation-induced interaction of phospholipid model membranes. Ph.D. Thesis. State University of New York at Buffalo

Düzgüneş, N., Hong, K., Papahadjopoulos, D. 1980. Membrane fusion: The involvement of phospholipids, proteins and calcium binding. *In:* Calcium Binding Proteins: Structure and Function. (F.L. Siegel, E. Carafoli, R.H. Kretsinger, D.H. MacLennan, and R.H. Wasserman, editors). pp. 17–22. Elsevier/North-Holland, New York

Düzgüneş, N., Ohki, S. 1977. Calcium-induced interaction of phospholipid vesicles and bilayer lipid membranes. *Biochim. Biophys. Acta* **467**:301

Düzgüneş, N., Wilschut, J., Fraley, R., Papahadjopoulos, D. 1981. Studies on the mechanism of membrane fusion: Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochim. Biophys. Acta (in press)*

Eisenberg, M., Gresalfi, T., Riccio, T., McLaughlin, S. 1979. Adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry* **18**:3213

Gresh, N. 1980. Intermolecular chelation of two serine phosphates by Ca^{2+} and Mg^{2+}. A theoretical structural investigation. *Biochim. Biophys. Acta* **597**:345

Hammoudah, M.M., Nir, S., Isac, T., Kornhauser, R., Stewart, T.P., Hui, S.W., Vaz, W.L.C. 1979. Interactions of La^{3+} with phosphatidylserine vesicles: Binding, phase transition, leakage and fusion. *Biochim. Biophys. Acta* **558**:338

Hauser, H., Dawson, R.M.C. 1967. The binding of calcium at lipid-water interfaces. *Eur. J. Biochem.* **1**:61

Hoekstra, D., Yaron, A., Carmel, A., Scherphof, G. 1979. Fusion of phospholipid vesicles containing a trypsin-sensitive fluorogenic substrate and trypsin. *FEBS Lett.* **106**:176

Jacobson, K., Papahadjopoulos, D. 1975. Phase transitions and phase separations in phospholipid vesicles, induced by changes in temperature, pH and concentration of divalent metals. *Biochemistry* **14**:152

Kantor, H.L., Prestegard, J.H. 1975. Fusion of fatty acid containing lecithin vesicles. *Biochemistry* **14**:1790

Kurland, R.J., Hammoudah, M., Nir, S., Papahadjopoulos, D. 1979*a*. Binding of Ca^{2+} and Mg^{2+} to phosphatidylserine vesicles: Different effects on P-31 NMR shifts and relaxation rates. *Biochem. Biophys. Res. Commun.* **88**:927

Kurland, R., Newton, C., Nir, S., Papahadjopoulos, D. 1979*b*.

Specificity of Na^+ binding to phosphatidylserine vesicles from a ^{23}Na NMR relaxation rate study. *Biochim. Biophys. Acta* **551**:137

Martin, F.J., MacDonald, R.C. 1976. Phospholipid exchange between bilayer membrane vesicles. *Biochemistry* **15**:321

McLaughlin, A., Grathwohl, C., McLaughlin, S. 1978. The adsorption of divalent cations to phosphatidylcholine bilayer membranes. *Biochim. Biophys. Acta* **513**:338

McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., McLaughlin, A. 1981. The adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* (*in press*)

McLaughlin, S.G.A., Szabo, G., Eisenman, G. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* **58**:667

Miller, C., Arvan, P., Telford, J.N., Racker, E. 1976. Ca^{2-}-induced fusion of proteoliposomes: Dependence on transmembrane osmotic gradient. *J. Membrane Biol.* **30**:271

Newton, C., Pangborn, W., Nir, S., Papahadjopoulos, D. 1978. Specificity of Ca^{2+} and Mg^{2+} binding to phosphatidylserine vesicles and resultant phase changes of bilayer membrane structure. *Biochim. Biophys. Acta* **506**:281

Nir, S. 1977. Van der Waals interactions between surfaces of biological interest. *Prog. Surface Sci.* **8**:1

Nir, S., Bentz, J. 1978. On the forces between phospholipid bilayers. *J. Colloid Interface Sci.* **65**:399

Nir, S., Bentz, J., Portis, Jr., A.R. 1980*a*. Effect of cation concentrations and temperature on rates of aggregation of acidic phospholipid vesicles. Application to fusion. *Adv. Chem.* **188**:75

Nir, S., Bentz, J., Wilschut, J. 1980*b*. Mass action kinetics of phosphatidylserine fusion as monitored by coalescence of internal vesicle volumes. *Biochemistry* **19**:6030

Nir, S., Newton, C., Papahadjopoulos, D. 1978. Binding of cations to phosphatidylserine vesicles. *Bioelectrochem. Bioenerg.* **5**:116

Ohki, S., Düzgüneş, N. 1979. Divalent cation-induced interaction of phospholipid vesicle and monolayer membranes. *Biochim. Biophys. Acta* **552**:438

Ohki, S., Sauve, R. 1978. Surface potential of phosphatidylserine monolayers. I. Divalent ion binding effect. *Biochim. Biophys. Acta* **511**:377

Ohnishi, S.-I., Ito, T. 1974. Calcium-induced phase separations in phosphatidylserine-phosphatidylcholine membranes. *Biochemistry* **13**:881

Papahadjopoulos, D. 1968. Surface properties of acidic phospholipids: Interaction of monolayers and hydrated liquid crystals with uni- and bi-valent metal ions. *Biochim. Biophys. Acta* **163**:240

Papahadjopoulos, D., Miller, N. 1967. Phospholipid model membranes. I. Structural characteristics of hydrated liquid crystals. *Biochim. Biophys. Acta* **135**:624

Papahadjopoulos, D., Nir, S., Ohki, S. 1972. Permeability properties of phospholipid membranes: Effect of cholesterol and temperature. *Biochim. Biophys. Acta* **266**:561

Papahadjopoulos, D., Portis, A., Jr., Pangborn, W. 1978. Calcium-induced lipid phase transitions and membrane fusion. *Ann. N.Y. Acad. Sci.* **308**:50

Papahadjopoulos, D., Poste, G., Schaeffer, B.E., Vail, W.J. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim. Biophys. Acta* **352**:10

Papahadjopoulos, D., Vail, W.J., Jacobson, K., Poste, G. 1975. Cochleate lipid cylinders: Formation by fusion of unilamellar lipid vesicles. *Biochim. Biophys. Acta* **394**:483

Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G., Lazo, R. 1977. Studies on membrane fusion. III. The role of calcium-induced phase changes. *Biochim. Biophys. Acta* **465**:579

Papahadjopoulos, D., Vail, W.J., Pangborn, W.A., Poste, G. 1976. Studies on membrane fusion. II. Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. *Biochim. Biophys. Acta* **448**:265

Portis, A., Newton, C., Pangborn, W., Papahadjopoulos, D. 1979. Studies on the mechanism of membrane fusion: Evidence for an intermembrane Ca^{2+} phospholipid complex, synergism with Mg^{2+}, and inhibition by spectrin. *Biochemistry* **18**:780

Seimiya, T., Ohki, S. 1973. Ionic structure of phospholipid membranes, and binding of calcium ions. *Biochim. Biophys. Acta* **298**:546

Sun, S., Day, E.P., Ho, J.T. 1978. Temperature dependence of calcium-induced fusion of sonicated phosphatidylserine vesicles. *Proc. Nat. Acad. Sci. USA* **75**:4325

Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R., Hagins, W.A. 1977. Liposome-cell interactions: Transfer and intracellular release of a trapped fluorescent marker. *Science* **195**:489

Wilschut, J., Düzgüneş, N., Fraley, R., Papahadjopoulos, D. 1980. Studies on the mechanism of membrane fusion: Kinetics of Ca ion induced fusion of phosphatidylserine vesicles followed by a new assay for mixing of aqueous vesicle contents. *Biochemistry* (*in press*)

Wilschut, J., Düzgüneş, N., Papahadjopoulos, D. 1981. Calcium/magnesium specificity in the kinetics of aggregation and fusion of phosphatidylserine vesicles: Role of vesicle size. *Biochemistry* (*in press*)

Wilschut, J., Papahadjopoulos, D. 1979. Ca^{2+}-induced fusion of phospholipid vesicles monitored by mixing of aqueous contents. *Nature* (*London*) **281**:690

Received 24 June 1980