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Effects of Divalent Cations, Temperature, Osmotic Pressure Gradient, and Vesicle Curvature on Phosphatidylserine Vesicle Fusion

Shinpei Ohki
Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, New York 14214

Summary. Fusion of phosphatidylserine vesicles induced by divalent cations, temperature and osmotic pressure gradients across the membrane was studied with respect to variations in vesicle size. Vesicle fusion was followed by two different methods: 1) the Tb/DPA fusion assay, whereby the fluorescent intensity upon mixing of the internal aqueous contents of fused lipid vesicles was monitored, and 2) measurement of the changes in turbidity of the vesicle suspension due to vesicle fusion. It was found that the threshold concentration of divalent cations necessary to induce vesicle fusion depended on the size of vesicles; as the diameter of the vesicle increased, the threshold value increased and the extent of fusion became less. For the osmotic pressure-induced vesicle fusion, the larger the diameter of vesicles, the smaller was the osmotic pressure gradient required to induce membrane fusion. Divalent cations, temperature increase and vesicle membrane expansion by osmotic pressure gradient all resulted in increase in surface energy (tension) of the membrane. The degree of membrane fusion correlated with the corresponding surface energy changes of vesicle membranes due to the above fusion-inducing agents. The increase in surface energy of 9.5 dyn/cm from the reference state corresponded to the threshold point of phosphatidylserine membrane fusion. An attempt was made to explain the factors influencing fusion phenomena on the basis of a single unifying theory.

Key Words vesicle fusion · surface energy · divalent cations · osmotic pressure gradient · temperature · membrane curvature

Introduction

In recent years a number of membrane fusion studies have been made using model membranes, to elucidate the mechanism of biological membrane fusion [29]. So far, several proposals regarding the mechanism of membrane fusion in such systems have been put forward. The first of them was that "membrane micellization" contributed to membrane fusion, as proposed by Lucy and co-workers [1, 2, 20]. Membrane micellization in the membrane would be caused by membrane destabilizing molecules, such as lysolecithin and short-chain fatty acids, and micellized membrane portions in two membranes in close contact may be responsible for membrane fusion. A similar view (but with a membrane semi-micelle configuration) has been held by Breisblatt and Ohki [4]. From temperature-induced membrane fusion and divalent cation-induced membrane fusion studies, these authors also proposed that the increased hydrophobicity (surface tension) of a membrane surface is the main factor in membrane fusion [5]. Papahadjopoulos and co-workers have stressed the importance of lateral segregation of acidic lipids due to divalent cations [25, 27]. Fusion is thought to occur at the boundaries between the domain of acidic lipid-divalent cation complexes and the remaining liquid crystalline lipid membrane phase. The formation of a nonbilayer type structure (e.g. hexagonal lipid) for phospholipid membranes containing phosphatidylethanolamine and some acidic phospholipids in the presence of Ca^{2+} has been observed by these authors [8, 9]. Cullis and coworkers proposed that such an inverted micelle structure at the region of contact of two opposing membranes could become an intermediate state for membrane fusion [35, 36]. Hui et al. [15], Schullery et al. [31] and Lichtenberg et al. [19] have worked on the fusion of neutral phospholipid membranes and proposed that the phase defect sites, which may arise from different phase behavior of mixed lipid components or which may result from nonuniform phase configurations at a low temperature, may be the sites to induce membrane fusion and stated that such configurations are not necessary for the formation of inverted micelles. Recently, Portis, et al. [28], and Ekerdt and Papahadjopoulos [14] have proposed the importance of a transmembrane-Ca^{2+} complex as the site of direct interaction between the two opposed membranes.

Our theory that the increased hydrophobicity of the membrane surface is responsible for membrane fusion has been further investigated by us for various membrane systems; 1) in a study of

fusion between phosphatidylserine vesicles and phosphatidylcholine monolayer membranes, induced by divalent cations, it is demonstrated that the higher the surface tension of a phosphatidylcholine monolayer, the lower is the threshold concentration of divalent cations required to induce fusion [24]; 2) the surface energy increase due to membrane expansion with increased temperature correlates well with the temperature dependence of membrane fusion [6]; 3) the surface energy increase of a monolayer due to the divalent cations in the subphase also correlates with the concentration dependence of membrane fusion induced by divalent ions [22].

In this paper, the theory will be further tested on the membrane fusion properties of various sizes of vesicles with respect to divalent cation concentrations, increased temperature and osmotic pressure gradients. The degree of membrane fusion induced by the above agents will be correlated with the corresponding surface energy changes of the vesicle membrane due to the same agents. (Some of the results obtained in this study were presented previously (Ohki, 1983).)[1]

Materials and Methods

Chemicals

Phosphatidylserine was purchased from Avanti Biochemical Co. (Birmingham, Ala.). Some of the purchased lipids were further purified through a DEAE (Diethylaminoethyl) cellulose column by a slightly modified method of Rouser et al. [30]. Both samples showed a single spot on silica gel thin-layer chromatographic plates. Each lipid sample gave identical experimental results within experimental errors.

NaCl was of ultrapure grade, purchased from Alfa Chemical Co. Before use, the NaCl was roasted at 400 to 500 °C for 2 hr to eliminate possible organic contaminants. $TbCl_3 \cdot 6\,H_2O$ (99.9% pure) and dipicolinic acid (pyridine-2,6-dicarboxylic acid) were obtained from Alfa and Sigma Chemical Companies, respectively. Buffers used were a mixture of L-histidine (Calbiochem, A grade) and Tes (Calbiochem, Ultrol grade). Hexane was used as the lipid solvent. It was obtained from Fluka (Purum Grade) and further purified through activated alumina and silica gel (Fisher Chemical Co.) columns to remove possible contaminating surfactants. Other chemicals used were reagent grade, obtained from Fisher Chemical Co. Some solutions contained a small amount of EDTA (0.01 mM) to remove possible divalent and polyvalent cation contaminants in the experimental solutions. The water used was distilled three times, including an alkaline permanganate process.

Small Unilamellar Vesicle Preparation

Small unilamellar vesicles were prepared in either (a) 100 mM NaCl, (b) 138 mM sucrose/10 mM NaCl, (c) 10 mM $TbCl_3$ and 100 mM sodium citrate or (d) 100 mM dipicolinic acid (DPA) (sodium salt) solution, all containing 2 mM L-histidine and 2 mM Tes as buffer. The pH of these salt solutions was adjusted to 7.4 with NaOH. The phosphatidylserine was dispersed in one of the above aqueous solutions at a concentration of 10 μmol/ml, then vortexed for 10 min, sonicated for 1 hr and centrifuged at 100,000 × *g* as described in an earlier paper [13]. The yield of small (~250 Å diameter) unilamellar vesicles was about 95% of the total lipid used for preparation. Vesicles prepared in (c) and (d) solutions were separated from nonencapsulated materials using a Sephadex G-75 column according to the method of Wilschut et al. [38, 40]. The elution buffer (0.1 M NaCl/2 mM histidine/2 mM Tes) contained 0.05 mM EDTA.

Large Unilamellar Vesicle Preparation

Large unilamellar vesicles were prepared by a method modified from the Reverse Phase Evaporation (REV) method [32]. Phosphatidylserine was dissolved in chloroform (2.5 mg lipid/ml) and about 13% (vol/vol) of aqueous solution (b), (c), or (d) was added to the above lipid-chloroform solution; the mixture was sonicated for 10 min; was then evaporated almost completely to dryness and the remaining mixture was hydrated by 1 ml of the same aqueous solution per 10 μmol phospholipids, and shaken gently to form a completely uniform milky suspension. This suspension was then passed through a Sepharose CL-2B column (about 30 cm length × 1.6 cm diameter, under 20 to 30 cm H_2O pressure) to fractionate different size distributions of vesicles. Each elution sample was collected from the column in 2-ml aliquots. The mean size of vesicles in each fraction was determined by negative staining electron microscopy (Hitachi, HU-11). Within each 2-ml fraction, there was to some extent a distribution of vesicle sizes, but the average size for two successive fractions was distinctly different.

Assay of Vesicle Fusion

The fusion of small and large unilamellar vesicles induced by divalent cation or temperature was followed by a Tb/DPA assay, monitoring the fluorescent intensity (SLM-8000 spectrofluorimeter, SLM Instruments) due to mixing of the internal aqueous contents of unilamellar vesicles. The details are described in an earlier paper [22]. For divalent-cation-induced membrane fusion, the temperature of the sample solution was maintained at 23 °C. After $TbCl_3$ and DPA-encapsulated vesicles of an equimolar amount (0.1 μmol phospholipid each for small vesicles and 0.05 μmol each for larger vesicles) were suspended in 2 ml of NaCl buffer solution in a quartz curvette, divalent cations (1 M $CaCl_2$ or $MgCl_2$) were injected into the vesicle suspension in small increments, and the solution was well shaken to give a homogeneous mixture. It took 10 sec to change the divalent ion concentration in the experimental solution. The fluorescent intensity was measured about 15 sec after each change in divalent ion concentration. The excitation wavelength was 275 nm and the emission fluorescence was measured at 545 nm employing a Corning 3–68 cut-off filter to eliminate the contribution of light scattering to the signal. The value for 100% fusion was determined in the presence of 50 μM dipicolinic acid by release of the contents from Tb-containing vesicles (the same amounts as in the experiment, except free from EDTA during the Sephadex G-75 column chromatography) with 0.5% (wt/wt) sodium cholate. The experiments were performed at 23 °C.

For temperature-induced vesicle fusion, the same method was used to detect fusion as outlined above. After two kinds of small unilamellar vesicles (containing Tb and DPA, respectively) were suspended in 0.1 M NaCl buffer solution, the vesi-

[1] A part of this paper was presented at the 27th Annual Meeting of the Biophysical Society, San Diego, California, February, 1983.

cles were aggregated by addition of 3.5 mM Mg^{2+}. At this concentration, small phosphatidylserine vesicles (~250 Å diameter) did aggregate extensively but did not fuse to any appreciable extent even after 40 min at 23 °C. After the addition of 3.5 mM $MgCl_2$ to the vesicle suspension, it was left for 10 min to attain a near equilibrium for vesicle aggregation, and then the temperature of the suspension solution was raised gradually at the rate of approximately 1.5 °C per min.

Temperature-induced fusion was also measured by monitoring turbidity (at 400 nm) of a small unilamellar vesicle (~250 Å in diameter) suspension with use of a double-beam spectrophotometer (Hitachi 100-60). The lipid vesicles were preaggregated by 3.5 mM $MgCl_2$ at 23 °C and then the temperature was raised at the same rate as in the above-mentioned temperature-induced fusion experiments. When the vesicle suspension reached a certain temperature, EDTA at twice the concentration of Mg^{2+} was added to the suspension in order to disperse aggregated vesicles; then the residual turbidity of the suspension was measured. The residual turbidity corresponded to a measure of irreversible changes in the state of the lipid vesicles; we assumed that the magnitude of such residual turbidity corresponded to the degree of vesicle membrane fusion. In order to obtain a vesicle fusion-temperature relationship, each experimental point corresponding to one temperature was obtained by performing several trial experiments as described above.

The fusion of large unilamellar vesicles induced by osmotic pressure gradients across the membrane was detected by measuring the turbidity, as described above. Vesicles of large average diameter were aggregated by 2 mM Mg^{2+} in 0.138 M sucrose and 10 mM NaCl. According to fluorescence assays these vesicles did not fuse at this Mg^{2+} concentration. Small amounts of the preaggregated vesicle suspension were transferred to each of several solutions of different osmotic strengths (0.1 μmol PL/2 ml): (a) sucrose 0.138 M and 10 mM NaCl, (b) 0.104 sucrose and 10 mM NaCl, (c) 0.0675 M sucrose and 10 mM NaCl, (d) 0.0334 mM sucrose/10 mMNaCl and (e) 0 mM sucrose/10 mM NaCl. All of these solutions contained 2 mM Mg^{2+}, 1 mM histidine and 1 mM Tes, and the pH of the solutions was 7.4.

The preaggregated vesicle suspension transferred to solution (a) did not show any change in turbidity with time. However, the preaggregated vesicle suspension transferred to any of the hypotonic solutions (b), (c), (d) or (e) showed a time-dependent change in turbidity. The more hypotonic the solution, the greater the time of gradual change in turbidity lasted. After the gradual change in turbidity ceased, EDTA (at 2 times the Mg^{2+} concentrations) was added to the solution and the turbidity was measured. Since the turbidity of such vesicle suspensions also depended on the tonicity of the solutions (e.g. sucrose concentrations), a measurement similar to the above was made for vesicle suspensions in the absence of Mg^{2+} as a control experiment; the net change in the residual turbidity with and without Mg^{2+} upon the addition of EDTA was taken to be a measure of membrane fusion. The turbidity change due to the change in vesicle volume was similar to that observed by the earlier workers [11]. The experiments were performed at 23 °C.

Threshold of Vesicle Fusion

For divalent cation-induced vesicle fusion, the fluorescence intensity measured 15 sec after each change of divalent ion concentration was plotted as a function of divalent ion concentration. The concentration at which the fluorescence intensity-concentration curve gave the sharpest increase, was determined. This was defined as the "threshold concentration" of divalent cation inducing vesicle fusion. A reduction in fluorescence intensity with time was observed at divalent ion concentrations above the threshold value, but this was not observed below the threshold concentration of divalent ions. For temperature-induced vesicle fusion, the similar definition of "threshold" as the above was used with the fluorescent intensity-temperature curve. For the fusion assay by turbidity measurements, the point where the residual turbidity gave a significant large increase, was taken as the threshold point for fusion, although this measure may not entirely be dependable as an appropriate base for the threshold event.

Surface Tension Measurements

Phosphatidylserine monolayers were prepared by placing an aliquot of the lipid spreading solution (approximately 1 mM lipid in hexane) by means of a microsyringe (Hamilton) on an aqueous surface of a constant area (64 cm^2 in a glass dish). The surface tension was measured after complete evaporation of hexane. The area per molecule of each monolayer was kept constant at 65 $Å^2$. Subphase solutions were 0.1 mM NaCl or 0.01 M NaCl containing a small amount of buffer (2 or 1 mM histidine and 2 or 1 mM Tes) and 0.01 mM EDTA. The pH of solutions was adjusted to 7.4 with NaOH. The surface tension of the monolayers was measured with an electronic balance (Beckman), using a microscopic cover glass (18 × 18 × 0.2 mm) as a Wilhelmy plate (accuracy of ±0.1 dyn/cm). The procedure for measuring the surface tension was almost the same as that published earlier [24]. The surface tension of a phosphatidylserine monolayer corresponding to the above area per molecule was reproducible within 2 dyn/cm for each monolayer. The experiments were designed to measure the change in the surface tension of phosphatidylserine monolayers as a function of divalent cation concentration in the subphase solution. Divalent cation concentrations were altered by injecting a small amount of a concentrated salt solution (1 M of $CaCl_2$ or $MgCl_2$) to the subphase solution. After each injection of salt, the solution was stirred well with a magnetic stirrer. The experiments were performed at 23 °C.

Monolayer Expansion Study

Monolayers were formed by spreading an aliquot of the lipid sample on the surface of 0.1 M NaCl buffer solution, with or without 3.5 mM $MgCl_2$ in a Langmuir trough. The dimension of the Teflon trough was 27 × 6 cm^2. A Teflon bar was used as the moving bar for compression or expansion of the monolayer.

The surface tensions of an aqueous phase and monolayers were measured by the Wilhelmy plate method mentioned above. The temperature of the solution in the trough was varied by circulating a mixture of antifreeze and water through a coil in the subphase solution. The temperature of the circulating mixture was controlled by a combination of a cooler (Haake model FK) and water circulator (Haake model FS). The experimental temperature was that of the subphase solution, which was accurate to within ±0.5 °C. The monolayer was formed initially at low pressure (~5 dyn/cm) at room temperature (23 °C), and then the temperature of the subphase solution was varied from 1 or 2 degrees above the phase transition (gel-liquid crystalline phases) to the fusion temperature [6]. The phase transition temperatures are 7 °C for phosphatidylserine membrane in 0.1 M NaCl solution and about 15 °C for 0.1 M NaCl/3.5 mM $MgCl_2$ solution. The latter value of the phase transition temperature was obtained by using the equation proposed by Traüble and Eibl [34] and the experimental results for the PS membrane by Jacobson and Papahadjopoulos [16]. The monolayer was then compressed to 47 dyn/cm and the temperature

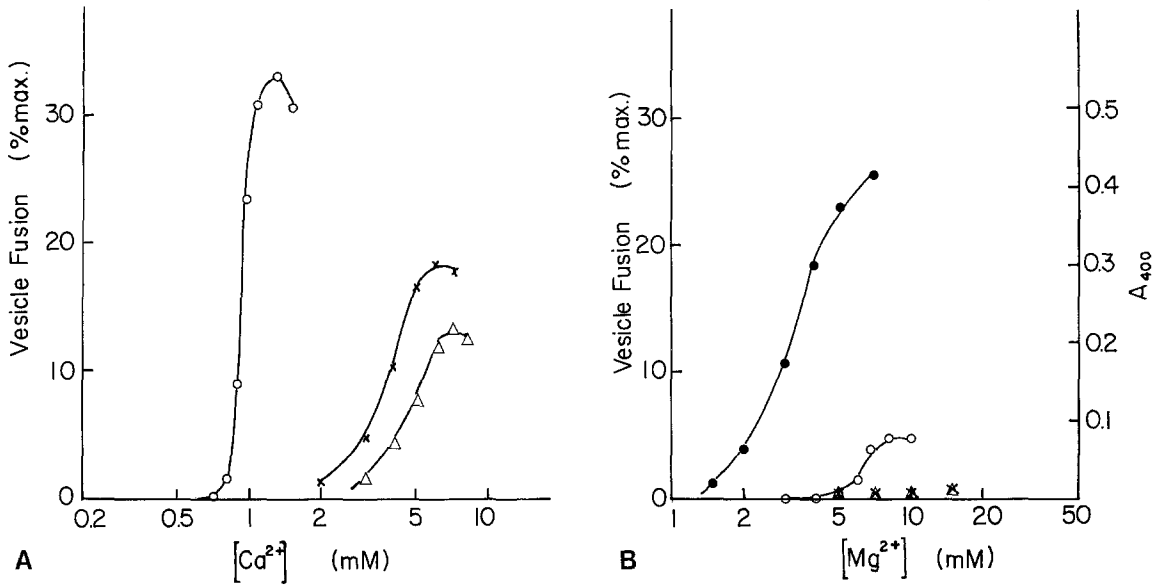

Fig. 1. (*A*): Fusion of phosphatidylserine vesicles of various sizes in 0.1 M NaCl with respect to Ca^{2+} concentrations. Two kinds of phospholipid vesicles (0.1 μmol lipid each for 250 Å diameter vesicle or 0.05 μmol lipid each for large diameter vesicles) which are encapsulated with 10 mM Tb/100 mM sodium citrate and 100 mM dipicolinic acid, respectively, were suspended in 2 ml 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA, pH 7.4. The ordinate refers to the fluorescence intensity at 545 nm and the abscissa refers to Ca^{2+} concentration. The intensity is expressed in percentage of the maximum fusion. The wavelength of excitation light was 275 nm. ○: average 250 Å diameter vesicles; ×: average 2,000 Å diameter vesicles; △: average 10,000 Å diameter vesicles. (*B*): Similar plots of the experimental results as described in *A* except for the use of Mg^{2+} instead of Ca^{2+}. ○: average 300 Å diameter vesicles; ×: average 2,000 Å diameter vesicles; △: average 10,000 Å diameter vesicles. ●: indicates the turbidity of small (250 Å diameter) vesicles of phosphatidylserine suspended in 0.1 M NaCl buffer solution (0.1 or 0.05 μmole lipid/2 ml) with respect to Mg^{2+} concentration. The right-hand ordinate refers to the turbidity A_{400}

was increased keeping the film surface tension constant (47 dyn/cm), in which the effect of temperature on water surface tension is included.

Experimental Results

Figure 1 shows the changes in fluorescence intensity of phosphatidylserine vesicle suspensions having different average size vesicles, with variation of divalent cation concentrations; here two kinds of phospholipid vesicles which contained encapsulated $TbCl_3$ or dipicolinic acid, respectively, were suspended in 0.1 M NaCl/2 mM His/2 mM Tes/0.01 mM EDTA, pH 7.4. The change in fluorescence intensity corresponds to the degree of fusion of the two kinds of vesicles. The threshold concentrations of Ca^{2+} required to induce vesicle membrane fusion (as defined above) were 0.9 mM for the vesicle suspension of average diameter 250 Å size vesicles (approximately the same values as those obtained by others [22, 38], 4.0 mM for the 2000 Å average diameter vesicles, and 5.0 mM for the 1 μm average diameter vesicles (Fig. 1*A*). The threshold concentration of Mg^{2+} was 6.5 mM for the 250 Å diameter vesicles but, for the larger size vesicle ($\geqq$2000 Å in diameter) suspensions, there was no significant change in fluorescence intensity observed up to 15 mM Mg^{2+} and there was no observable threshold concentration of Mg^{2+} (Fig. 1*B*). This observation corresponds to that reported by others [39]. Each data point in the above and following Figures represents the average of four or more experiments.

The increase in surface tension of a phosphatidylserine monolayer (65 $Å^2$/molecule) is shown as a function of divalent cation concentration in Fig. 2. The monolayer was formed on an aqueous solution of 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA, pH 7.4. As seen in Fig. 2, the surface tension increases as the Ca^{2+} or Mg^{2+} concentration in subphase solutions is increased. At the threshold concentration of 0.9 mM Ca^{2+} for 250 Å diameter vesicle suspension, the surface tension increase would correspond to 7.7 dyn/cm and at the threshold concentration of 5.0 mM Ca^{2+} for 1 μm diameter vesicle suspension, the surface tension increase would correspond to about 9.5 dyn/cm. The surface tension increases with the Ca^{2+} concentration until a saturation point is reached. For Mg^{2+}, the surface tension increase approaches a value slightly lower than 8 dyn/cm. At 6.5 mM Mg^{2+}, which corresponds to the Mg^{2+} concentra-

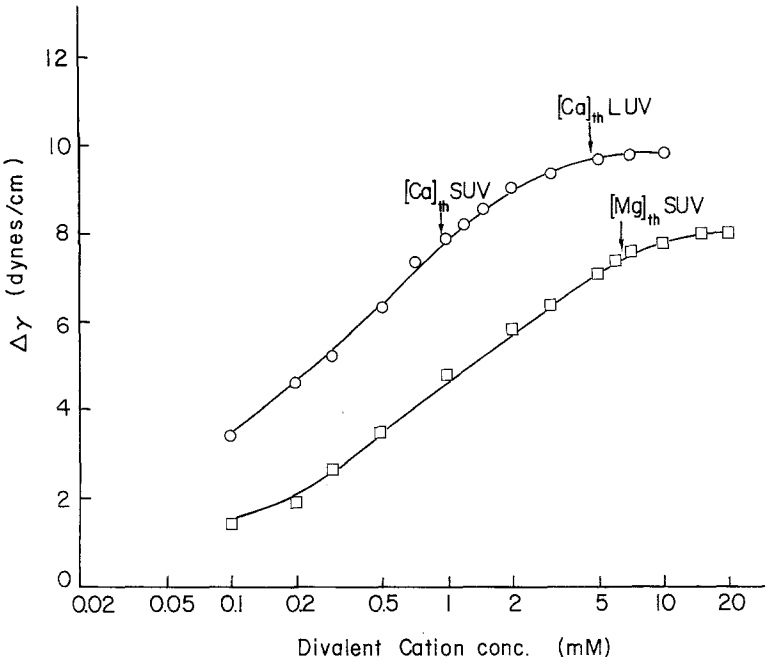

Fig. 2. Surface tension increases in a phosphatidylserine monolayer (65 Å^2 per molecule) formed at the air-water interface with respect to divalent cations (Ca^{2+} or Mg^{2+}) concentrations. The subphase solution consisted of 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA, pH 7.4. The initial surface tension $\gamma(C_2=0)$ of a phosphatidylserine monolayer of 65 Å^2 per molecule was 45 ± 2 dyn/cm and $\Delta\gamma \equiv \gamma(C_2)-\gamma(C_2=0)$ where C_2 refers to the divalent ion concentrations. o: Ca^{2+} and □: Mg^{2+}

tion for the fusion threshold of 250 Å diameter vesicles, the surface tension change is about 7.5 dyn/cm. A further increase in Mg^{2+} concentration in the subphase does not change the surface tension as much as an increase in Ca^{2+} concentration does; saturation appears to be reached below 8 dyn/cm.

The degree of fusion of phosphatidylserine vesicles with increase in temperature is shown in Fig. 3. In this case, only small unilamellar (250 Å diameter) vesicles were used in the experiment; the vesicles were suspended in 0.1 M NaCl/2 mM histidine/2 mM Tes/0.01 mM EDTA, pH 7.4. Then 3.5 mM $MgCl_2$ was added to the vesicle suspension in order to aggregate vesicles and the suspension was left for 10 min at room temperature 23 °C before the temperature was increased. The temperature of the vesicle suspension was increased at a rate of about 1.5 °C per min after the $MgCl_2$ was added. Vesicle fusion was not observed until the temperature was approximately 45 °C. The threshold temperature for fusion due to increased temperature was 50 °C. It was assured from a control experiment that the measured increase in fluorescence intensity was not due to the temperature effect on the fluorescent intensity. Within a slight variation in the rate of increase in temperature (1 to 2 °C per min), fusion was not significantly affected by different rates of temperature increase. It was also observed that the fluorescence intensity has a maximum at about 60 °C, and above 60 °C, the intensity either became saturated or decreased gradually. It was also observed that there was no

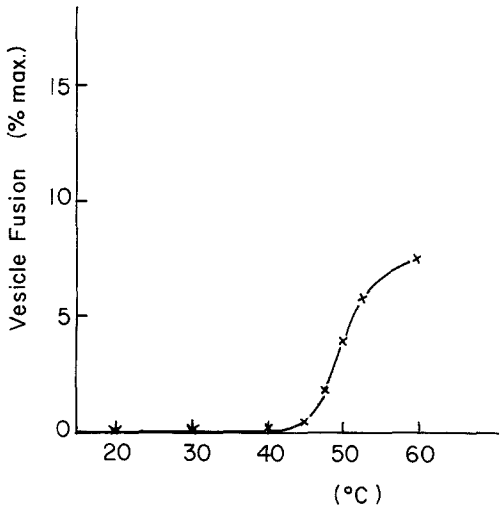

Fig. 3. Small average (250 Å diameter) unilamellar phosphatidylserine vesicle fusion induced by temperature. Vesicle fusion was monitored by Tb/DPA fluorescence assay technique similar to that in Fig. 1. The vesicles were suspended in 0.1 M NaCl buffer (0.1 M NaCl/2 mM His/2 mM Tes, pH 7.4) solution in the presence of 3.5 mM Mg^{2+}. The temperature was raised at the rate of 1.5 °C per min

significant fusion of vesicles at 3.5 mM Mg^{2+} for 40 min at 23 °C. Also, in the absence of Mg^{2+} no fusion of vesicles was observed up to 60 °C for 30 min.

As another method to study the temperature-induced vesicle fusion, the turbidity of the vesicle suspension was measured with respect to increase in temperature where lipid vesicles were suspended at a concentration of 0.1 μmol/ml in 0.1 M NaCl

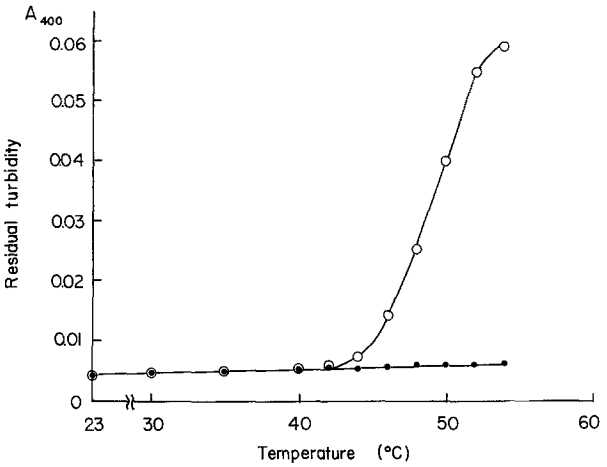

Fig. 4. Fusion assay by turbidity measurements. The irreversible change in turbidity of small unilamellar phosphatidyl vesicle (250 Å diameter) suspension with respect to increased temperature. o indicates the irreversible change in turbidity of vesicles which were preaggregated by the addition of 3.5 mM Mg^{2+}, upon the addition of 7 mM EDTA in the suspension at each experimental temperature. • refers to the case where no Mg^{2+} was present in the suspension solution. 0.1 μmol phospholipid/ml of 0.1 M NaCl/2 mM His/2 mM Tes, pH 7.4

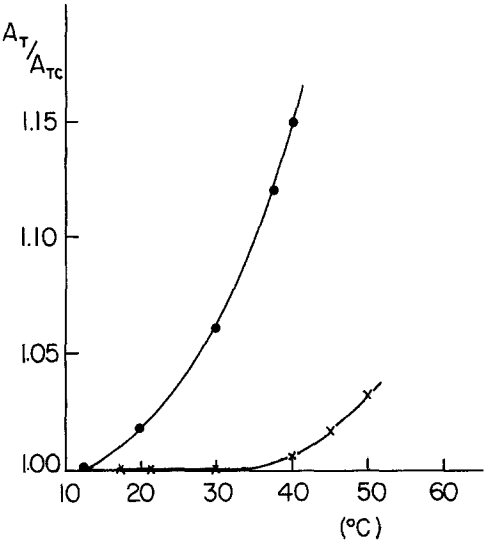

Fig. 5. Expansion of phosphatidylserine monolayers formed at the air/water interfaces with respect to the increase of temperature. The subphase was 0.1 M NaCl/2 mM His/2 mM Tes, pH 7.4 with or without 3.5 mM Mg^{2+}. The ratio of the area per molecule at certain temperature to that just above the phase transition temperature (gel-liquid crystalline: 8 °C for no Mg^{2+}; 18 °C for 3.5 mM Mg^{2+} cases) was plotted against temperature, keeping a constant surface tension (47 dyn/cm). •: in 0.1 M NaCl without Mg^{2+}; x: in 0.1 M NaCl with 3.5 mM Mg^{2+}

buffer solution and then 3.5 mM $MgCl_2$ was added to the suspension in order to have a preaggregated vesicle suspension as described above. The experimental results (Fig. 4) showed that the behavior of the temperature-induced vesicle fusion curve

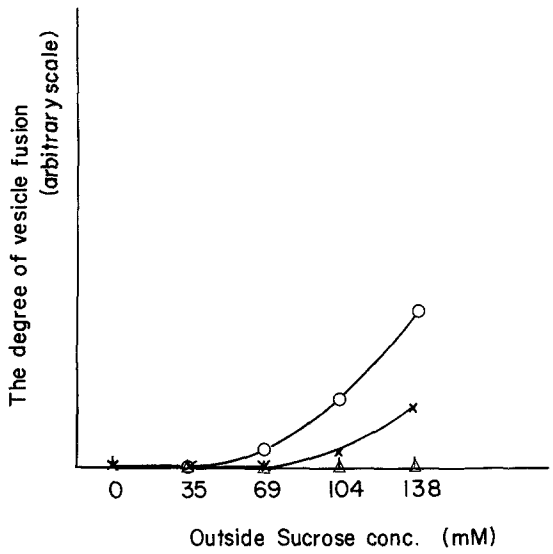

Fig. 6. Relative degree of membrane fusion of phosphatidylserine vesicles of various sizes, induced by osmotic pressure gradient. The vesicles prepared in 0.138 M sucrose/0.01 M NaCl/1 mM His/1 mM Tes, pH 7.4 and then preaggregated in the presence of 2 mM $MgCl_2$ in the suspension solution, were suspended in various experimental solutions (0.1 μmol phospholipid in 2 ml experimental solution): 0.138 M sucrose ($\Delta C=0$), 0.104 M sucrose ($\Delta C=0.035$ M), 0.069 M sucrose ($\Delta C=0.069$ M), 0.0345 M sucrose ($\Delta C=0.104$ M) and 0 mM sucrose ($\Delta C=0.138$ M) all containing 10 mM NaCl, 2 mM $MgCl_2$, 1 mM His, 1 mM Tes, pH 7.4. The degree of fusion was measured as the irreversible turbidity change of the suspension after the addition of 5 mM EDTA in the suspension. o: the vesicles of the average diamter of 1 μ; x: the vesicles of the average diameter of 6,000 Å; △: vesicles of the average diameter of 1,000 Å

was very similar to those obtained by the fluorescence fusion assay. Vesicle fusion did not occur until approximately 45 °C, although the turbidity of the suspension solution started to increase at 35 °C, and the threshold temperature of fusion was approximately 50 °C.

Figure 5 shows the expansion of a phosphatidylserine monolayer with increasing temperature, where the monolayer was formed on the same NaCl buffer solution as in temperature-induced vesicle fusion. In the case where no Mg^{2+} was in the subphase solution, the expansion rate was greater than in the case where 3.5 mM Mg^{2+} was present, and at 37 °C the monolayer expanded 1.12 times with respect to the area per molecule at 8 °C, under the condition of a constant surface tension (47 dyn/cm). When 3.5 mM Mg^{2+} was in the subphase solution, the monolayer did not expand as much as when there was no Mg^{2+}; under the same surface tension (47 dyn/cm) the monolayer expanded only 3% from 18 to 50 °C. The latter temperature corresponded to that for temperature-

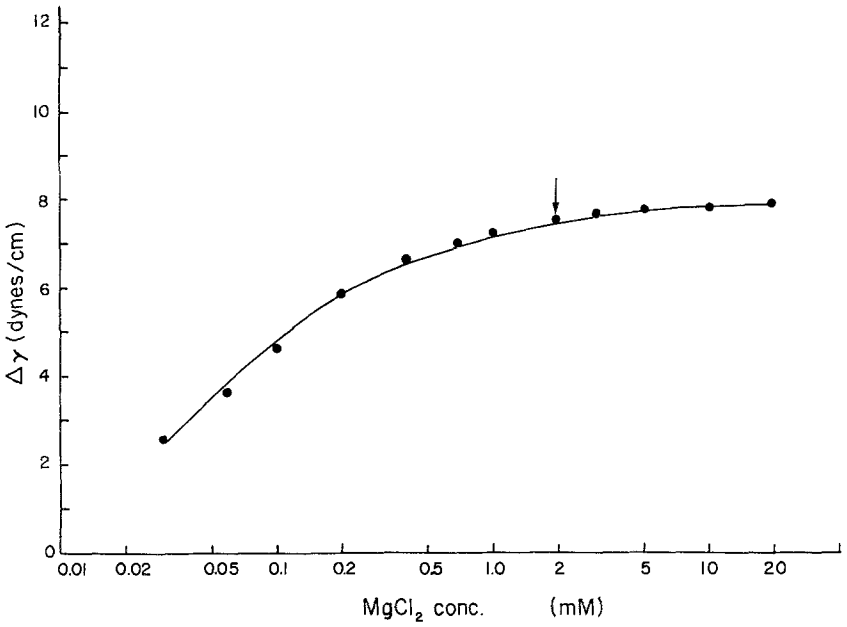

Fig. 7. Surface tension increase in the phosphatidylserine monolayer (65 Å^2 per molecule) formed at the air/water interface with respect to various Mg^{2+} concentrations. The subphase solution consisted of 0.01 M NaCl/2 mM His/2 mM Tes/0.01 mM EDTA, pH 7.4. The initial surface tension $\gamma(C_2=0)$ of a phosphatidylserine monolayer of 65 Å^2 per molecule was 45 ± 2 dyn/cm and $\Delta\gamma \equiv \gamma(C_2)-\gamma(C_2=0)$ where C_2 refers to Mg^{2+} concentration. The arrow in the Figure shows the surface tension increase of the monolayer at the concentration (2 mM Mg^{2+}) where the vesicles in the suspension were preaggregated

induced membrane fusion of phosphatidylserine vesicles in the presence of 3.5 mM Mg^{2+}.

The degree of fusion of phosphatidylserine vesicles of different average size distributions with respect to the variation of osmotic pressure gradients applied across the vesicle membrane was studied as another experiment. As mentioned in Materials and Methods, the fusion was defined as the net residual turbidity change in vesicle suspensions after the addition of EDTA to the aggregated suspension. The experimental results (Fig. 6) showed that the larger the diameter of the vesicles, the smaller the osmotic pressure gradient (outside being more hypotonic than inside) across a vesicle membrane was needed for fusion. For the 1.0-μm average diameter vesicles, the threshold osmotic pressure gradient required to induce vesicle fusion was 0.104 M in sucrose, for the 0.6-μm average diameter vesicles it was 0.138 M in sucrose, and for the 0.1-μm average diameter vesicles, there was no appreciable fusion even at a gradient of 0.138 M in sucrose.

Figure 7 shows the surface tension increase of phosphatidylserine monolayers (area = 65 Å^2/molecule) with increase of $MgCl_2$ concentration in the subphase, where the monolayer was formed on 0.01 M NaCl/1 mM His/1 mM Tes/0.01 mM EDTA, pH 7.4. The arrow indicates the concentration (2 mM Mg^{2+}) at which phosphatidylserine vesicles were preaggregated in the suspension solution used for the osmotic gradient-induced fusion study. At this concentration, the surface tension increase of the monolayer was 7.6 dyn/cm. In the presence of sucrose (0.1 M) in addition to the above salt concentration in the subphase solution, the increase in surface tension with respect to Mg^{2+} concentration was approximately the same as the above. Each data point in all of the above Figures represents the average of four or more experiments and its standard error is within 5% of the value.

Discussion

As seen in the Experimental Results, the degree of vesicle fusion due to divalent cations or osmotic pressure gradients varies with vesicle size; for fusion induced by divalent cations, as the size of vesicles increases, the threshold concentration of divalent cation increases and the extent of fusion becomes less. This is consistent with observations made by others [18, 39]. On the contrary, for the osmotic pressure-induced vesicle fusion, the larger the diameter of vesicle, the smaller is the osmotic pressure gradient required to induce membrane fusion (of vesicles preaggregated by Mg^{2+}).

We have proposed in an earlier work [22] that the degree of membrane fusion induced by divalent cations corresponds to an increase in surface free energy of the interacting membranes; at the threshold concentrations of divalent cations required to induce fusion of phosphatidylserine vesicles, the increased surface tensions of monolayers of the same phospholipid are the same, for all cations tested. How would this be applicable to the present study of membrane fusion induced by divalent cations of phosphatidylserine vesicles of various sizes?

The main point to consider is that the surface tension of a phospholipid monolayer is, because of curvature effects, less than that of a vesicle, if both are in the same salt environment. Large vesicles (average diameter greater than 0.2 μ) will have a sufficiently large radius of curvature for their surface tensions to approximate that of a planar monolayer. Small vesicles (average diameter about 250 Å) will have a greater surface tension than that of the larger vesicles in the same salt solution. Thus, the surface tensions measured for a planar monolayer, at the respective threshold concentrations of divalent ions for vesicle fusion, are 7.7 dyn/cm for the situation corresponding to small vesicle (250 Å average diameter) fusion and 9.5 dyn/cm for that to large vesicle fusion; this difference of 1.8 dyn/cm may be rationalized as a contribution due to curvature to the surface tension of the small vesicles. Moreover, this explanation is consistent with the results that the monolayer surface tension due to Mg^{2+} did not exceed 8 dyn/cm even at 15 mM Mg^{2+}, but approached a limiting value just below 8 dyn/cm, and that correspondingly, large phosphatidylserine vesicles (>1000 Å in diameter) failed to fuse even at a Mg^{2+} concentration up to 15 mM, while small vesicles (250 Å) fused to a small extent at 6.5 mM Mg^{2+}; the curvature may, at this Mg^{2+} concentration, give an additional increment in surface energy (~1.8 dyn/cm) which is barely sufficient to cause fusion of the small vesicles (~7.5 dyn/cm + 1.8 dyn/cm ≅ 9.3 dyn/cm).

In the case of membrane fusion induced by osmotic pressure gradients, the following should be taken into account. Since the osmotic pressure gradient was applied such that the internal pressure was greater than the outside, the vesicle volume would increase and, in turn, the membrane surface would expand. When small amounts of vesicle suspension, with vesicles preaggregated by Mg^{2+}, were suspended in a hypotonic solution, a gradual time-dependent turbidity change was observed. This tendency was greater with greater hypotonicity of the suspension. However, this gradual turbidity change ceased within a few minutes even for the highest tonicity difference used in this experiment ($\Delta C = 0.138$ mM sucrose). This turbidity change can be attributed to the swelling of vesicles due to the osmotic pressure difference across the vesicle membrane. The experimental data for the net turbidity change was taken at 5 min after the preaggregated vesicles were suspended in each hypotonic solution. Therefore, the measurements were made under conditions close to mechanical equilibrium (i.e. $\Delta\pi = \Delta P$).

Now, consider what the increase in surface free energy of the vesicle membrane would be when an osmotic pressure difference is applied across the membrane. In order to estimate such a surface energy increase, we assume that the application of an osmotic pressure difference expands the membrane, i.e. the vesicle swells. This membrane expansion due to the osmotic pressure difference results in an increase in surface free energy sufficient to induce membrane fusion. In the discussion of fusion induced by divalent cations, we deduced that an increase in surface energy of 9.5 dyn/cm relative to a reference state (the membrane surface in the presence of only NaCl buffer solution) is sufficient for membrane fusion. For a large unilamellar vesicle (>0.2 μm in diameter), the outside and inside membrane surfaces can be considered, approximately, as planar. Therefore, the surface energy increase in the outer surface of a large vesicle may be about 7.6 dyn/cm for preaggregated vesicles in the presence of 2 mM Mg^{2+}, according to the experimental results for a planar monolayer membrane, shown in Fig. 7; the inside surface, however, would still have nearly the same surface tension as the reference state. In order for fusion to occur, it would be necessary for the surface energy of the outside vesicle surface to be increased additionally by about 1.9 dyn/cm. Such a surface tension increase would correspond to a 4.0% expansion of the area per molecule in the membrane; in this estimate we have taken the energy increase due to membrane expansion to be given by the following simple formulation [23]:

$$\gamma A = \gamma_o A_o + \gamma_{o/w} \Delta A \tag{1}$$

where A and A_o are the areas per molecule of the membrane at the expanded and reference states, respectively, and we assume a value of 65 Å per molecule for A_o; ΔA is the increased area per molecule; γ and γ_o are the surface tensions of the expanded and reference states, respectively, where γ_o is about 1 dyn/cm [33]. $\gamma_{o/w}$ is the surface tension of the oil-water interface which is about 50 dyn/cm [10]. Thus, an additional increase of 1.9 dyn/cm in the surface tension of the outer membrane surface over that of 7.6 dyn/cm due to the presence of 2 mM Mg^{2+}, requires an expansion of about 2.5 $Å^2$ per molecule [i.e. 4% expansion according to Eq. (1)]. The work done in this membrane expansion is expressed by:

$$W = \int \bar{\gamma}(A)\, dA + \int \gamma(A)\, dA \tag{2}$$

where $\bar{\gamma}$ and γ are the surface tension of the inner and outer surfaces of the vesicle membranes, re-

spectively. In this case, $\bar{\gamma}(A_o) \neq \gamma(A_o)$, because the external surface is in the presence of a NaCl buffer solution with 2 mM Mg^{2+}, while the inner membrane surface is in the presence of a NaCl buffer solution with no Mg^{2+}. However, we assume that $\bar{\gamma}(A) - \bar{\gamma}(A_o)$ is approximately equal to $\gamma(A) - \gamma(A_o)$, a difference of approximately 1.9 dyn/cm at the threshold point of vesicle fusion.

Now, W shown in Eq. (2) should be equal to the work done by an osmotic pressure gradient in the vesicle expansion from the reference state to a new equilibrium, expanded state:

$$W = \iint \Delta P(t)\, dA\, dr = \iint \Delta P(r)\, dA\, dr, \qquad (3)$$

where $\Delta P(t)$ is the time-dependent pressure difference which will be equal to $\Delta\pi = RT\Delta C$ at equilibrium state; $\Delta P(t)$ can also be expressed as a function of the radius r of a vesicle. Here, we make an approximation that $\gamma(A)$ and $\Delta P(r)$ have, respectively, a linear dependence on A, the area per molecule, and r, the radius of the vesicle. We can then evaluate the integrals in Eqs. (2) and (3). The surface tension increases for different osmotic gradients across a vesicle membrane which would result in a 4% expansion per molecule are tabulated in Table 1. If our model is correct, the experimental threshold value of the osmotic pressure gradient for vesicle fusion should correspond to the case where the theoretical value shows a surface tension increase of 1.9 dyn/cm in Table 1. The experimental observations and theoretical values agree qualitatively (Table 2).

The membrane fusion due to osmotic pressure gradients has also been studied for small lipid vesicle systems in the presence of Ca^{2+} [21] and for multilamellar lipid vesicles interacting with planar lipid bilayer membranes [7]. Their results qualitatively support our present experimental results. It should be noted that the latter study may indicate that osmotic gradients across the planar membrane enhances protein transfer from vesicle to the planar membrane, not necessarily as a result of membrane fusion.

It is also interesting to mention that cells or relatively large biological membrane vesicles (erythrocytes [12], synaptosomes [37], etc.) are susceptible to osmotic pressure gradients and can be lysed easily by them, but small vesicles (synaptic vesicle, etc.) are relatively stable even with large osmotic pressure gradients [37]. This corresponds with our present findings.

The above model can also be used to interpret the results of temperature-induced membrane fusion (Figs. 3 and 4). The results show that small

Table 1. Surface energy increases resulting from the expansion of vesicle membranes by osmotic pressure gradients

ΔC	Vesicle diameter		
(mM)	1 μm (dyn/cm)	0.6 μm (dyn/cm)	0.1 μm (dyn/cm)
150	2.81	1.72	0.31
138	2.58	1.55	0.26
104	1.94	1.16	0.16
69	1.24	0.78	0.13
34	0.97	0.39	0.07
0	0	0	0

Table 2. Threshold osmotic pressure gradients and the corresponding energy increases of membrane surface

Vesicle size (diameter-μm)	Experimental threshold ΔC (mM)	Calculated value of energy increase by the threshold ΔC (dyn/cm)	Total energy increase relative to the reference state (dyn/cm)
1	~100	1.87	7.6+1.87=9.47
0.6	~138	1.55	7.6+1.55+Δ =9.15+Δ[a]
0.1	–	–	–

[a] Δ indicates an extra energy due to a smaller curvature of the vesicle

unilamellar phosphatidylserine vesicles in 0.1 M NaCl buffer containing 3.5 mM $MgCl_2$ fuse when the temperature is raised from 20 to 50 °C. Moreover, a phosphatidylserine monolayer on a corresponding aqueous subphase solution shows a 3% expansion in area per molecule when the temperature of subphase solution is increased from 18 to 50 °C (Fig. 5). The surface energy of small unilamellar vesicles in 0.1 M NaCl/3.5 mM $MgCl_2$ solution is 6.5 dyn/cm higher than that in the reference state according to the monolayer surface tension studies. The expansion of 3% would correspond to an increase in surface energy of 1.4 dyn/cm from Eq. (1). Therefore, the total increase in surface energy of small unilamellar vesicles at 50 °C relative to the reference state (the monolayer on 0.1 M NaCl solution at 20 °C) would be 6.5 dyn/cm (due to the presence of 3.5 mM Mg^{2+}) plus 1.4 dyn/cm (due to the temperature-induced expansion) plus 1.8 dyn/cm (due to the curvature of the small vesicles), for a total of 9.7 dyn/cm. This estimate could be somewhat high, since the thermal expansion of bilayer (vesicles) membranes may not be as great as that for the monolayer system.

We have thus demonstrated a qualitative correspondence between our theoretical model for mem-

brane fusion and the experimental observations in three different types of experiments, in the sense that a strong correlation between the degree of membrane fusion and the increase in surface free energy brought about by apparently dissimilar agents – curvature, divalent cations, temperature, osmotic pressure gradients, – is found. Although the process of membrane fusion induced by divalent cations, temperature increase or an osmotic pressure gradient may appear to be different in their molecular pathways, all have a common feature: a similar increase in the surface free energy (or increased hydrophobicity) of the membrane appears to be required for membrane fusion.

This model for membrane fusion qualitatively can accommodate various membrane fusion models proposed by others mentioned in the Introduction. The surface of a semi-micelle configuration [4, 20] in the membrane should have a higher free energy surface than the planar surface of the membrane. The boundary regions between two molecular phases (liquid crystalline-gel phases) [27] , because of the irregular molecular arrangements probably occurring at these regions, could have higher surface energy states such as in semi-micelle configuration than the bulk membrane surface of homogeneous (regular) molecular arrangements. The structural defect due to molecular segregation or restriction of molecular packing due to temperature of different molecular species [15, 31] would create higher free energy surfaces (more hydrophobic area) in such localized regions. Membrane fusion due to polyethylene glycol (PEG) [17] may also correspond to the similar energy situation where the interaction of PEG with a membrane would increase the surface energy of the membrane.

It should be noted that the fusion of phospholipid membranes containing phosphatidylethanolamine or similar molecules [8, 9, 35] may proceed on a pathway quite different from that for phosphatidylserine. For the latter, fusion may be associated with a high free energy state of the lipid polar group region, whereas, for the former, fusion may correlate with a higher energy state of the liquid hydrocarbon (hydrocarbon interior) region relative to the polar group region, which tends to form inverted micelle structures. A recent study has indicated that most of such inverted micelle structures are obtained at equilibrium long after fusion occurred but not during the membrane fusion processes [3].

The model outlined here for a mechanism of membrane fusion applies to the case where the membrane surface is considered as homogeneous and undergoes a uniform change in physical properties. For fusion which may occur through molecular interaction between specific sites at the membrane surfaces as may well be the case for fusion of biological membranes, the theory needs some modification.

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