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Monovalent Cation-Induced Phospholipid Vesicle Aggregation: Effect of Ion Binding†

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ABSTRACT: Aggregation of acidic phospholipid vesicles induced by monovalent cations was studied for vesicles of small and large sizes. It was found that there were two phases in the aggregation of large acidic phospholipid vesicles. In the initial phase, observed in the range of 0.1–0.4 M monovalent salts, aggregation took place spontaneously after a change in salt concentration; in the second phase (>0.4 M salt), aggregation progressed gradually with time. The order of capability for monovalent cations to induce the initial phase of aggregation of large phosphatidylserine vesicles (more than 1000 Å in diameter) was $Li^+ > Na^+ > K^+ > TEA^+$. However, for the second phase of aggregation, the order was $Na^+ > Li^+ > K^+ > TEA^+$, which was the same as that to induce massive aggregation of small phosphatidylserine vesicles (250 Å in diameter). A similar reversal in the order was observed in studies of the surface potential of the phosphatidylserine monolayer. In these studies, the order of the binding strength of monovalent cations was deduced from the change in surface potential produced by successive additions of $MgCl_2$ to the subphase solution, which contained a certain level of monovalent salt initially. These measurements were carried out with monolayers that had a range of areas per molecule. The order was $Na^+ > Li^+ > K^+$ for monolayers of large area (>80 $Å^2$) per molecule and was $Li^+ > Na^+ > K^+$ for those of small area (<80 $Å^2$) per molecule. For phosphatidic acid vesicles, the order of monovalent cations inducing vesicle aggregation was independent of the size of the vesicles and the monovalent salt concentration: $Na^+ > Li^+ > K^+ > Cs^+$. In this case, too, two phases of the aggregation process were observed. From the surface potential experiment of phosphatidic acid monolayers, it was also deduced that the adsorption of Na^+ on the phosphatidic acid membrane surface is slightly stronger than that of Li^+ in the range of areas (60~90 $Å^2$) per molecule. The observed phenomena of monovalent cation-induced vesicle aggregation with respect to variation of the size of vesicle as well as salt concentration were examined in terms of binding constant and accessibility of monovalent cations to the binding sites of lipid polar groups.

In order to elucidate biological membrane adhesion and fusion, a number of studies to mimic such phenomena using model membranes have been done for the last decade (Nir et al., 1983). Among them, it is shown that acidic phospholipid membranes can adhere or fuse in the presence of proper amounts of cations in membrane bathing solutions (Nir et al., 1983a,b; Papahadjopoulos et al., 1974). The degree of such membrane adhesion or fusion seems to depend on the strength of binding of these ions to negatively charged polar groups of lipid molecules (Ohki, 1982). Although monovalent cations can induce aggregation of acidic phospholipid membranes (Ohki et al., 1982), they do not induce fusion of these membranes (Ohki, 1982), probably because of a rather weak binding capability of monovalent cation to phospholipid molecules when compared with those of divalent and polyvalent cations (Ohki, 1982; Düzgünes, et al., 1981). The presence of monovalent cations in the solution, however, does influence the degree of divalent cation binding as well as their fusion capabilities, probably due to a competitive binding to phospholipid polar group binding sites between monovalent and divalent cations (Ohki, 1982; Düzgünes, et al., 1981; Nir et al., 1983a,b).

Recently, we have studied phosphatidylserine vesicle aggregation induced by various monovalent cations (Ohki et al., 1982). It was found that the order of the capability of monovalent cations to induce aggregation of small vesicles made of phosphatidylserine molecules is $H^+ > Na^+ > Li^+ > K^+ > Cs^+ > TEA^+$. From this, we have deduced that the order of binding strength of these ions is the same as the above. However, others (Hauser et al., 1970; Eisenberg et al., 1979) have measured electrophoretic mobility of the large multilamellar vesicles of the same lipid in various monovalent salt suspension and found that the order of the binding of these ions is $H^+ > Li^+ > Na^+ > K^+ > Cs^+ > TEA^+$, which is almost the same as the above except for the relative positions of Na^+ and Li^+ in the series. The same order of ion binding strength was obtained for multilamellar phosphatidylserine membrane systems from the study with X-ray diffraction techniques (Loosley-Millman et al., 1982; Hauser & Shipley, 1983). We have suggested (Ohki et al., 1982) that these differences might be due to the use of different sizes of vesicles in the two different experiments.

In order to elucidate possible reasons for these observed differences, here we have performed experiments on vesicle

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aggregation induced by monovalent cations using various sizes of vesicles, some of which are comparable in size to those used in the above-referenced electrophoretic mobility experiments. Also, the surface potential studies on phospholipid monolayers with varying area per molecule and subphase monovalent salt species were performed in order to obtain the binding strength of monovalent cations to acidic phospholipid membranes as a function of area per molecule. The latter results are compared with those of the former (vesicle aggregation) studies on the strength of ion binding to the membrane surface as a function of various areas per lipid molecule (or size of vesicle). A possible molecular mechanism with respect to the interaction of monovalent cations to polar groups of acidic lipids is discussed.

Materials and Methods

Materials. Phosphatidylserine (bovine brain) (PS) and phosphatidic acid (derived from bovine brain phosphatidylcholine) (PA) were purchased from Avanti Biochemicals. Each lipid showed a single spot on silica gel thin-layer chromatographic plates. Ultrapure monovalent (K, Na, Li, Cs) salts in chloride form were obtained from Alfa Chemicals and used for monlayer experiments. Monovalent salts used in monolayer studies were roasted at about 600 °C for 1 h to eliminate contaminants of organic materials. Hepes [*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid] was the Ultrol grade from Calbiochem. All other chemicals used were of analytical reagent grade from Fisher Chemical Co. Water used was triply distilled including the process of alkaline $KMnO_4$ distillation.

Vesicle Preparation. Small unilamellar phospholipid vesicles were prepared by suspending phospholipids at a concentration of 10 μmol/mL of 0.1 M monovalent salt solution containing 2 mM Hepes, pH 7.0, vortexed for 10 min, and sonicated for 1 h in a bath-type sonicator (Heat Systems, Ultrasonics). Then, the suspension was centrifuged at 100000*g* for 1 h. The supernatant was used as a stock vesicle solution.

Large Unilamellar Vesicle Preparation. Large unilamellar vesicles were prepared by a method modified (Ohki, 1983) from the reverse-phase evaporation (REV) method (Szoka & Papahadjopoulos, 1978). Phospholipids were dissolved in chloroform (2.5 mg of lipid/mL) and about 13% (v/v) of aqueous buffer solution was added to the above lipid–chloroform solution; the mixture was sonicated for 10 min and was then evaporated almost completely to dryness, and the remaining mixture was hydrated by 1 mL of the same aqueous solution/10 μmol of 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 20–30 cm of 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 a distribution of vesicle sizes to some extent, but the average size of two sucessive fractions was distinctly different.

Turbidity Measurements. Turbidity of small unilamellar vesicle suspensions as a function of monovalent ion (Na^+, Li^+, K^+, and Cs^+) concentrations was measured at 400 nm by use of a Hitachi (100-60) spectrophotometer. The wavelength in the visible range at which sonicated unilamellar phosphatidylserine vesicles caused maximum light scattering was found to be approximately 400 nm. The vesicles were suspended at 0.1 μmol of phospholipid/mL in the salt solutions, and the ion concentrations were raised step by step by introducing small amounts of its concentrated salt solution (3∼5 M). The absorbance was measured 2 min after changing the ion concentration, and then the concentration was increased.

Turbidity of the large unilamellar vesicle suspensions as a function of monovalent ion concentrations was measured in the manner similar to the above except that the measurement was done at both 400 and 500 nm in order to account for the large size, but the relative magnitude of turbidity changes was the same for observations at both wavelengths. The vesicle concentrations were about 0.03∼0.08 mol/mL of suspension solutions.

Surface Potential Measurements. The surface potential of lipid monolayers was measured by use of an Americium air electrode (approximately 3 mm above the air/water interface) and a calomel pH reference electrode. The latter electrode was grounded and the ionizing air electrode was connected to the input of an electrometer (Keithley 610C). The output potential of the electrometer, which was related to "surface potential" (Davies & Rideal, 1961), was monitored by a strip chart recorder. The entire apparatus was shielded by a Faraday cage.

Lipid monolayers were formed on the aqueous phase (0.1 M monovalent salt solution) in a glass dish (9 cm in diameter) by spreading an aliquot of the hexane–lipid solution from a microsyringe (Hamilton). The subphase solution was either 0.1 M NaCl, LiCl, or KCl containing 2 mM Hepes, pH 7.0. Each experimental solution also contained some EDTA (at most 5 μM) to remove polyvalent cation contaminants possibly present in trace amounts in the systems. The concentration of phospholipids was determined by phosphate analysis (Bartlett, 1959). The monolayers used had various areas per molecule ranging from 60 to 90 Å^2 for the experiments. The value of area per molecule was also determined to be correct within 2 Å^2 by surface pressure (π) measurements.

In order to observe the surface potential change of monolayers as a function of ionic strength of the subphase solution and ion binding to the monolayers, $MgCl_2$ was injected into the subphase solution, while the monovalent salt concentration was kept constant (0.1 M). Then, a small amount of concentrated (3 M) $MgCl_2$ was injected into the monovalent salt hypophase of the monolayer from a microsyringe in order to attain a certain concentration of Mg^{2+}. The subphase solution was stirred well for homogeneous mixing. The successive addition of the concentrated $MgCl_2$ was made in order to obtain various concentrations of Mg^{2+} in the subphase solution.

Before the addition of salt, the aqueous subphase was stirred well, and it was ascertained that the monolayer surface potential was not altered by further stirring of the subphase solution. The standard mean error for the observed surface potentials were within ±1 mV. All experiments were done at room temperature of 24 ± 2 °C. Each data point represents the average of at least four experiments.

Results

Figure 1 shows the turbidity changes of large unilamellar phosphatidylserine vesicle (average diameter 3000 Å) suspensions with respect to changes in monovalent cation concentrations. There seems to be a two-phase increase in turbidity of vesicle suspension with an increase in monovalent salt concentration; the initial phase spans a monovalent salt concentration range of 0.1–0.5 M. In this range the turbidity change occurred instantaneously; in other words it showed a step increase with the change of salt concentration. In contrast, the second phase of turbidity change (for salt concentration >0.6 M) took place in a manner of the progressive increase with time after the salt concentration was increased in this

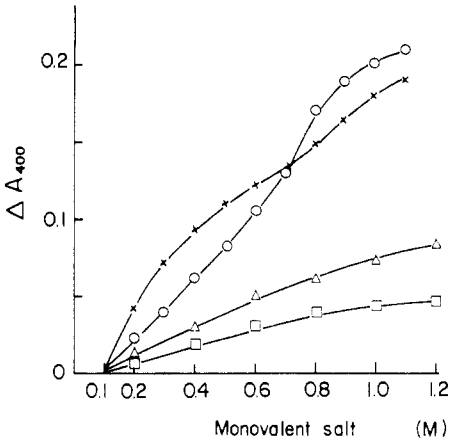

FIGURE 1: Turbidity (A_{400}) change ΔA of large (average 3000 Å in diameter) phosphatidylserine vesicles with respect to monovalent salt concentrations. The lipid concentrations were 0.03 μmol of PL/mL of salt solutions containing 2 mM Hepes and 0.05 mM EDTA, pH 7.0. (×) LiCl; (○) NaCl; (△) KCl; (□) CsCl.

range. In this case, it took about a half hour for the turbidity to reach a stationary value. However, in Figure 1, we have reported the turbidity values observed at the end of 2 min after each salt concentration change. The turbidity–salt concentration relationship showed a sigmoidal shape. This observation was similar to that seen for the small (~250-Å diameter) PS vesicle aggregation (Ohki et al., 1982). The turbidity increase is considered as a result of vesicle aggregation in the suspension solution since monovalent cations do not induce fusion of phosphatidylserine vesicles (Ohki, 1982). The order of effectiveness of monovalent cation to induce the inital spontaneous aggregation of large phosphatidylserine vesicles was found to be $Li^+ > Na^+ > K^+ > Cs^+$, in the region of monovalent salts up to 0.5 M. This is the same order of binding affinity of these ions for a large PS multilamellar vesicle membrane obtained from electrophoretic mobility studies (Hauser et al., 1970; Eisenberg et al., 1979). However, at higher concentration (> 0.6 M) the second phase of vesicle aggregation took place where Na^+ showed a greater effect than Li^+, but other monovalent cations remained in the same order of effectiveness as in the first phase of aggregation. The results similar to the above were also observed for the PS unilamellar vesicles having larger diameters than 3000 Å, although there was a slight change in the ratio of the magnitudes of turbidities between the initial and the second phases of aggregation; the relative magnitude of turbidity change for the initial phase of aggregation increased as the size of vesicles increased.

The first phase of aggregation of large PS vesicles was observed under a phase contrast microscope in the region of salt concentration of 0.2–0.5 M. The numbers and sizes of vesicle aggregates were progressively increased as the monovalent concentration was increased.

In order to show that the observation where Na^+ and Li^+ affinities inducing vesicle aggregation switch in order at higher salt concentration (>0.6 M) was not due to a possible difference in vesicle concentrations of different experimental solutions (since the difference in turbidity changes between Li^+ and Na^+ cases was not great), the large vesicles aggregated at 0.6 M NaCl or LiCl were sonicated to form small unilamellar vesicles to see how the turbidity changed. The turbidity, which was slightly greater for the Li^+ case than the Na^+ case (15% greater at 0.6 M LiCl than that at the same concentration of NaCl) for large unilamellar vesicles (3000 Å in diameter), was reversed (the turbidity ratio was reduced from 1.15 to 0.2) for small unilamellar vesicles. This confirms the

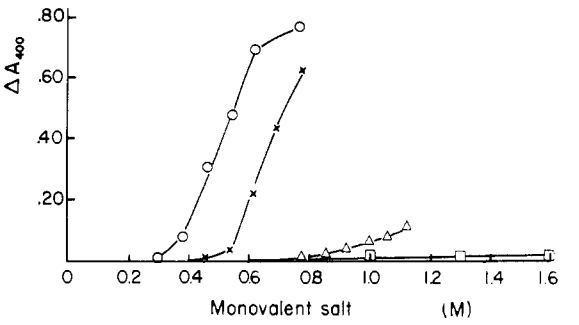

FIGURE 2: Turbidity (A_{400}) change ΔA of small (~250 Å in diameter) phosphatidic acid vesicles with respect to monovalent salt concentration. The lipid concentrations were 0.1 μmol of PL/mL of salt solutions containing 2 mM Hepes and 0.05 mM EDTA, pH 7.0. (×) LiCl; (○) NaCl; (△) KCl; (□) CsCl.

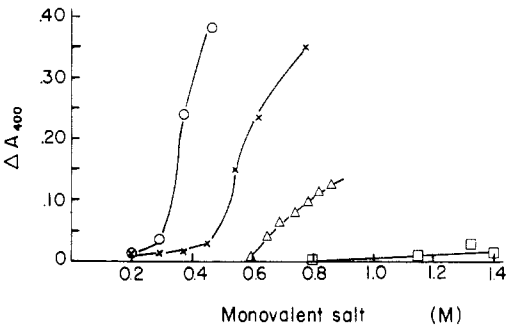

FIGURE 3: Turbidity (A_{400}) change ΔA of large (average 3000 Å in diameter) phosphatidic acid vesicles with respect to monovalent salt concentrations. The lipid concentration was 0.08 μmol of PL/mL of salt solutions containing 2 mM Hepes and 0.05 mM EDTA, pH 7.0. (×) LiCl; (○) NaCl; (△) KCl; (□) CsCl.

previous observation that the effectiveness of Na^+ in producing aggregation of the small (~250 Å in diameter) PS vesicles is greater than that of Li^+.

Experiments similar to the above but using phosphatidic acid were done for both small (250-Å diameter) and large (2000~4000-Å diameter) vesicles. The results are shown in Figures 2 and 3, respectively. In the case of phosphatidic acid, regardless of the size of vesicles, the order of effectiveness of monovalent cations to induce both the initial and the secondary aggregation of vesicles was the same as that for the small PS vesicle case: $Na^+ > Li^+ > K^+ > Cs^+$. The same observation was obtained for the case of large diameter (>4000 Å) vesicles. Monovalent cation concentrations required to produce a large and sharp change (the second phase aggregation) in turbidity of large PA vesicle suspensions were slightly lower than those for the large PS vesicles. For small PA vesicles (diameter 250 Å), such concentrations to produce a large and sharp change in turbidity of vesicle suspensions were also slightly lower than those for the small PS vesicle systems. Regardless of the size of vesicles, the aggregation kinetics of PA vesicles appeared to be similar to that of the small PS vesicles with respect to an increase in monovalent cation concentration; the change in turbidity occurred gradually with time after the salt concentration was increased. The turbidity vs. salt concentration relationship showed a sigmoid shape for both cases (large and small vesicles) (see Figures 2 and 3). However, a careful observation revealed that the first phase turbidity change (aggregation) was also observed for the large PA vesicles, but the extent of the turbidity change at this phase was much smaller than that for the large PS vesicles. In this case, the relationship between turbidity and salt concentration also has a monotonic shape. An important difference between the two cases (PS and PA vesicles) was that, at monovalent salt

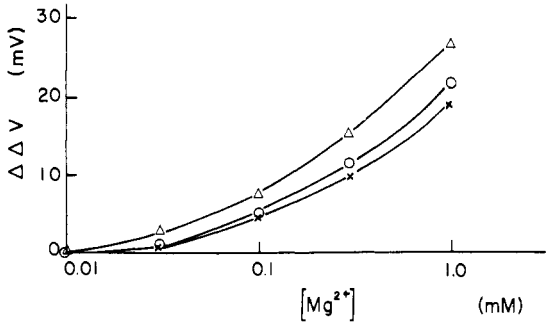

FIGURE 4: Surface potential changes ($\Delta\Delta V$) of phosphatidylserine monolayer vs. $MgCl_2$ concentrations in the subphase bulk solution. The monolayers having an area 65 Å^2 per molecule were formed at the air/water [0.1 M of monovalent salt (chloride salt, 2 mM Hepes, 5 μM EDTA, pH 7.0)] interface. (×) LiCl; (○) NaCl; (△) KCl.

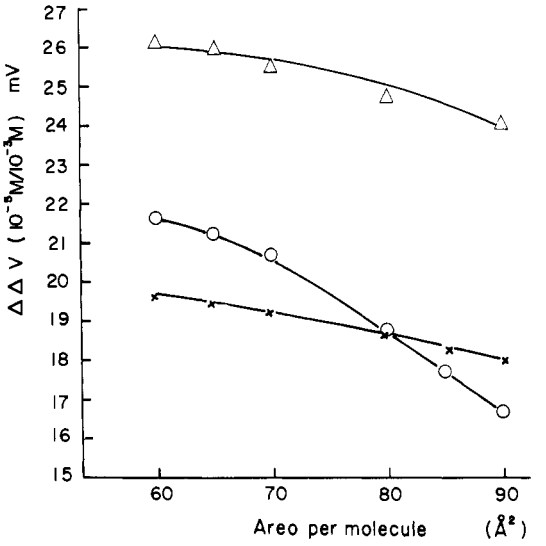

FIGURE 5: Surface potential changes, $\Delta\Delta V$ (10^{-5} M/10^{-3} M), of a phosphatidylserine monolayer for a Mg^{2+} concentration change of 10^{-5}–10^{-3} M at various areas per molecules. Subphase solutions are 0.1 M monovalent salt, 2 mM Hepes, and 5 μM EDTA, pH 7.0, having various $MgCl_2$ concentrations. (×) LiCl; (○) NaCl; (△) KCl.

concentrations less than 0.5 M, Na^+ affinity was greater than that of Li^+ for the large PA vesicles (see Figure 3) whereas Li^+ affinity was greater than Na^+ for the large PS vesicles (see Figure 1).

Figure 4 shows the surface potential changes ($\Delta\Delta V$) of a phosphatidylserine monolayer having an area of 65 Å^2 per molecule with respect to Mg^{2+} concentration in the presence of either 0.1 M NaCl, LiCl, or KCl in the subphase solution. The change in surface potential for a certain concentration change was in the order of $K^+ > Na^+ > Li^+$. This means that among these three monovalent cations used, K^+ is least effective to suppress Mg^{2+} binding to the site of the phosphatidylserine membrane at this area per molecule.

Experiments similar to the above were done with respect to PS monolayers having various areas per molecule (60–90 Å^2). The changes in surface potential of PS monolayers for Mg^{2+} concentration change from 10^{-5} to 10^{-3} M are shown in Figure 5 for each monovalent cation subphase solution as a function of area per molecule. At 60 Å^2 per molecule, the order of the change in surface potential was $K^+ > Na^+ > Li^+$, but at 90 Å^2 per molecule, the order changed to $K^+ > Li^+ > Na^+$. It is deduced from this experiment that the strength of Na^+ and Li^+ binding to phosphatidylserine membranes is altered as the area per molecule increases, provided that the Mg^{2+} binding constant is assumed to be unchanged.

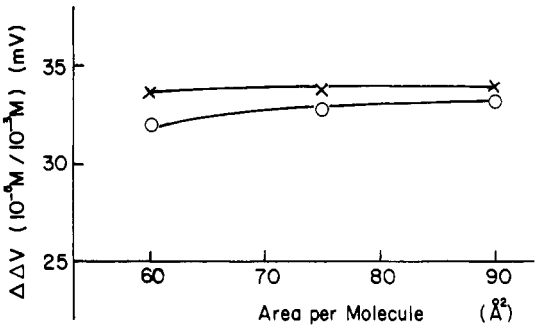

FIGURE 6: Experimental results similar to those in Figure 5 except for using a phosphatidic acid monolayer instead of a phosphatidylserine monolayer. (×) LiCl; (○) NaCl.

Monolayer experiments using phosphatidic acid instead of phosphatidylserine have also been done. The changes in surface potential of the phosphatidic acid monolayer for Mg^{2+} concentration change from 10^{-5} to 10^{-3} M at various area per molecule are shown in Figure 6 for both cases of 0.1 M NaCl and LiCl subphase solutions. It is seen from the figure that the change in surface potential is approximately constant regardless the variation of area per molecule of the monolayers and also the magnitude of the surface potential change for a given Mg^{2+} concentration change was much greater than those observed in the phosphatidylserine monolayer at the same condition. There was no significant difference in surface potential changes between the two cases of NaCl and LiCl subphases, although the magnitude of the potential change for the LiCl case was slightly greater than that for NaCl in all range of area per molecule examined.

Discussion

From the above experimental results, it may be considered that for a large phosphatidylserine vesicle membrane (>2000 Å in diameter), the order of binding of monovalent cations to the membrane is $Li^+ > Na^+ > K^+ > Cs^+$ in the range of monovalent salt concentration below 0.4 M. This order does correspond to that of the binding strengths of these ions to the PS membrane obtained from the electrophoretic studies using a large multilamellar PS vesicle suspended in 0.1 M monovalent salts by earlier workers (Hauser et al., 1970; Eisenberg et al., 1979). However, this order of binding appears to change to $Na^+ > Li^+ > K^+ > Cs^+$ at high concentration of monovalent salts (>0.6 M). This corresponds to that reported in our small vesicle aggregation studies (Ohki et al., 1982); the order of binding between Li^+ and Na^+ was reversed for small PS vesicles membranes. The surface potential measurements of a PS monolayer in this study also indicate the reversal of the order of Na^+ and Li^+ binding to a PS molecule as the area per molecule increases above 80 Å^2, the area of which may correspond to those for a small unilamellar vesicle membrane (Brouillete et al., 1982). The area 60–70 Å^2 per molecule may correspond to those for the large unilamellar vesicle membranes.

On the other hand, for phosphatidic acid vesicles, regardless of the size of vesicle, the order of effectiveness of monovalent cations in causing vesicle aggregation was $Na^+ > Li^+ > K^+ > Cs^+$, which was the same as that necessary to induce aggregation of the small PS vesicles. The ion binding affinity for the initial spontaneous aggregation for the large PA vesicles was also in the same order. The results of surface potential measurements of phosphatidic acid monolayers also indicate that the adsorption of Na^+ on the phosphatidic acid membrane surface is slightly stronger than that of Li^+. The large change in surface potential of the phosphatidic acid monolayer by

Mg^{2+} (Figure 6) than that for the phosphatidylserine monolayer (Figure 5) may be due to a greater binding constant of Mg^{2+} and smaller binding constants of monovalent cations (Na^+, Li^+) for the phosphatidic acid membrane than those for the phosphatidylserine membranes, respectively (S. Ohki and H. Oshima, unpublished results). These indicate that the binding affinity of Na^+ to a single phosphate group may be greater than that of Li^+. These observations would suggest that the accessibilities of the two ion binding sites (PO_4^-, COO^-) of a phosphatidylserine molecule to Li^+ and Na^+ may be different and varied depending on the size of a vesicle or the area per lipid molecule of the membrane. For the large PS vesicles, the area per molecule would be smaller than those of small vesicles; consequently, the space between two lipid polar groups would be smaller. In such a case, a small ion like Li^+ can penetrate and bind to the phosphate group easily that is located at the inner zone of the polar group region from the aqueous bulk phase, whereas larger ions like Na^+, K^+, and Cs^+ cannot penetrate into such a space easily. Therefore, when the area per molecule is smaller than a certain value (<80 Å^2) and the salt concentration is relatively low, the overall binding of cations to the PS lipid polar group sites (PO_4^-, COO^-) is such that Li^+ is more effective (in causing aggregation) than Na^+ and other monovalent cations, except for H^+. On the other hand, when the area per molecule becomes larger (>80 Å^2) i.e., the size of vesicle becomes smaller, the space between the two lipid polar heads could become greater, and consequently the inner binding site of the PS molecule, PO_4^-, is likely to be accessible to even a larger cation like Na^+ (as well as Li^+). In such cases, depending on the strengths of binding of Na^+ and Li^+ to the two different binding sites (PO_4^-, COO^-) of the lipid polar groups, the overall binding strengths of the two ions could be interchanged between Li^+ and Na^+ with the change of area per lipid of the membrane. A similar penetration of Na^+ into the PO_4^- site of the large PS membrane may occur when the concentration of Na^+ is sufficiently large (>0.6 M). This interpretation corresponds to the observation on the PA vesicle aggregation where the order of effectiveness of monovalent cations to induce aggregation is $Na^+ > Li^+ > K^+ > Cs^+$ regardless of the size of vesicle, because the accessible site for binding of a cation is only PO_4^-. For in PA, the site is not hindered by the other polar head group unlike the case for a PS membrane of small area per molecule, and also possibly the phosphate group binds more strongly to Na^+ than Li^+. The experimental result that a phosphatidylinositol (PI) membrane binds Na^+ more strongly than Li^+ (Eisenberg et al., 1979) corresponds well to the above interpretation.

Judging from the observed aggregation kinetics, the initial stage of aggregation (in concentration range of 0.2–0.4 M monovalent salts) of the large PS and PA vesicles may occur at the secondary minimum of the interaction potential energy profile defined in the DLVO theory (see Figure 7) because the aggregation occurs spontaneously after the change of monovalent salt concentration and reaches a stationary state. The interaction energies between two interacting vesicles can be calculated by using the equations described in the Appendix. The calculated interaction energy (Figure 7) shows that the depth at the secondary minimum energy point is about 1–3 kT for large vesicles (vesicle diameter >1000 Å) in the range of ionic concentration (0.3–0.6 M) observed for the initial aggregation by Na^+. The primary maximum is greater than 10 kT at 13-Å separation distance for 3000 Å diameter vesicles at 0.4 M NaCl. Therefore, it is energetically favorable for the two such vesicles to aggregate at the secondary minimum point but not at the primary minimum point in the energy

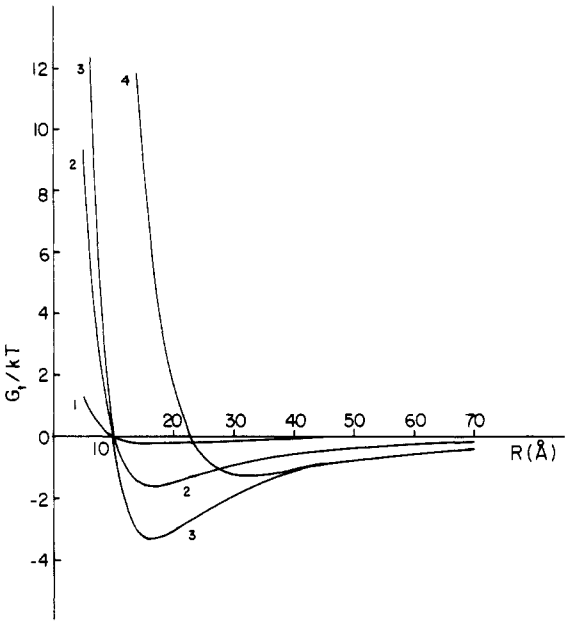

FIGURE 7: Total interaction energy between two PS vesicles (24 °C).

	vesicle diameter (Å)	binding constant of Na^+ (M^{-1})	NaCl concn (M)	S (Å^2)
curve 1	300	0.7	0.8	85
curve 2	1500	0.7	0.8	70
curve 3	3000	0.7	0.8	70
curve 4	3000	0.7	0.4	70

G_t = interaction energy between vesicles; R = separation distance between vesicles; A = Hamaker constant = 4×10^{-14} erg; h = thickness of vesicle membrane = 50 Å; S = area per lipid head group.

profile curve at such salt concentration. A similar argument concerning the possible aggregation of large lipid vesicles at the secondary minimum point has been made by Nir et al. (1981). However, at higher salt concentration, the primary maximum will be sufficiently small; for example, at 0.8 M NaCl, the barrier height of the primary maximum will be about 12 kT at 6-Å separation distance. In this concentration range, two large vesicles could stay at the secondary minimum point as well as the primary minimum point with a certain probability.

On the other hand, the interaction energy for the small PS vesicles (diameter 300 Å) indicates that even at high salt concentration (0.8 M NaCl), the depth of the secondary minimum for the two interacting small vesicles is much too small (less than 0.3 kT) to be able to maintain stable aggregates of the vesicles at this point but the primary maximum is about 1 kT at 6-Å interseparation distance of the two vesicles. Therefore, it is energetically possible for the two small vesicles to aggregate at the primary minimum relatively easily. This has also been pointed out by earlier workers (Nir & Bentz, 1978) for a small lipid vesicle interaction system. This may be the reason why the large vesicles aggregate spontaneously upon the increase of salt concentration (0.2–0.5 M), while the large vesicles in higher (>0.6 M) monovalent concentration range and the small vesicles aggregate gradually with time because in this case the two aggregating vesicles have to cross the primary energy barrier with a certain probability for each collision in order to fall in the primary minimum point. These also reflect on the shapes of turbidity vs. salt concentration curves; the large vesicles in the salt concentration range (0.2–0.5 M) aggregate in a manner of the first-order kinetics because the aggregation progresses monotonically with salt concentration similarly to the Michaelis–Menten kinetics, while

the large vesicles in high salt concentration range and the small PS vesicles aggregate in the higher order kinetics where the relationship between aggregation and salt concentration shows a sigmoid curve. The reaction kinetics showing such a relationship generally involves higher order kinetics in the reaction.

However, it is still difficult to give a satisfactory explanation for the different behaviors of aggregation kinetics between the large PS and PA vesicles, by use of the above-mentioned DLVO theory. The large PS and PA vesicles, both, aggregate spontaneously to a certain extent in the range of monovalent salt concentration of 0.2–0.4 M, but the magnitude of spontaneous aggregation of the large PA vesicles is smaller in this concentration range (0.2–0.4 M), and the ion binding order for inducing spontaneous aggregation of vesicles is different between PS and PA cases (i.e., $Li^+ > Na^+$ for PS and $Na^+ > Li^+$ for PA vesicles). Also, the concentrations inducing massive aggregation of large lipid vesicles are different for each case. In order to give satisfactory explanations for these, it may be necessary to add other factors in the simple DLVO equation used, such as the interaction energy terms due to the nature of surface hydrophilic layers that would vary with different phospholipids and have different ion binding properties to the different phospholipid polar groups of the membrane surface.

Recently, the effect of monovalent cations on divalent cation-induced phospholipid membrane fusion has been studied by Nir et al. (1983a,b), and it was observed that the order of monovalent cations to suppress the divalent cation-induced membrane fusion was $Li^+ > Na^+ > K^+$. It should be noted, however, that according to our recent work (Ohki, 1982; S. Ohki and H. Ohshima, unpublished results) the membrane fusion seems to be related not only to the amount of ions bound to the membrane surface but also more importantly to the change in surface nature, such as surface free energy due to dehydration of membrane surface or deformation of membrane molecule due to ion binding. It may be possible that Li^+ binds to polar groups and dehydrates more than Na^+ does (Hauser & Shipley, 1983), but the adsorbed amounts of ions to a certain membrane surface may be more for Na^+ than Li^+. In this regard, more detailed studies concerning the binding sites and the nature of binding of these ions to lipid membrane surfaces (Cevc et al., 1981) should be done in the future. The studies considering these factors are being undertaken in this laboratory in connection with elucidation of molecular mechanisms of cation-induced vesicle aggregation and fusion.

Appendix

Let us suppose that the vesicle membrane surface is negatively charged and adsorbs ions. We assume that one Na^+ ion can bind with one phospholipid and the binding of Cl^- is negligible. The surface charge density σ of the vesicle is then expressed (Ohki et al., 1982) as

$$\sigma = \frac{\sigma^{int}}{1 + Kn \exp[-e\psi_s/(kT)]} \tag{1}$$

where $\sigma^{int} = -e/S$ is the surface charge density with no Na^+ binding, e the elementary electric charge, S the average area occupied by one head group of the phospholipid on the vesicle surface, K the binding constant of Na^+, n the NaCl concentration, ψ_s the surface potential of the vesicle, k the Boltzmann constant, and T the absolute temperature.

The electrostatic interaction energy $G^{el}(R)$ due to the surface charges of two vesicles of radius r at the closest separation R between their surfaces can be calculated approximately from the corresponding interaction energy $G^{el}_{pl}(l)$ between two parallel plates at separation l per unit area (Derjaguin, 1934), viz.

$$G^{el}(R) = \pi r \int_R^\infty G^{el}_{pl}(l)\, dl \tag{2}$$

To obtain the value of $G^{el}_{pl}(l)$, we use the one-dimensional Poisson–Boltzmann equation for the electric potential $\psi(x)$ ($0 \lesssim x \lesssim l$) in an electrolyte solution between the two plates, viz.

$$\frac{d^2\psi}{dx^2} = \frac{8\pi en}{\epsilon} \sinh\left(\frac{e\psi}{kT}\right) \tag{3}$$

which must satisfy eq 1 [where $\psi_s = \psi(0)$] together with

$$\left.\frac{d\psi}{dx}\right|_{x=0} = -\frac{4\pi\sigma}{\epsilon} \tag{4}$$

where ϵ is the dielectric constant of the solution. Equation 4 ignores the influence of electrostatic fields inside the vesicle; this influence is indeed small for $\epsilon'/(\epsilon\kappa h) \sim 2 \times 10^{-3} \ll 1$ (Ohshima, 1974), where ϵ' is the dielectric constant of the lipid membrane, κ is the Debye–Hückel parameter defined as

$$\kappa = \left(\frac{8\pi ne^2}{\epsilon kT}\right)^{1/2} \tag{5}$$

an h is the membrane thickness.

$G^{el}_{pl}(l)$ can be calculated by solving numerically the simultaneous equations (1), (3), and (4), which will then give $G^{el}(R)$ via eq 2. Exact calculations of $G^{el}_{pl}(l)$, similar to those of Ohshima & Mitsui (1978) for the Ca^{2+} adsorption to the lipid membrane, show that for our experimental conditions [in Figure 6, $\kappa^{-1} \approx 3 \sim 4$ Å and $R \gtrsim 6$ Å ($\kappa R \gtrsim 2$)], $G^{el}(R)$ can be approximated by (with negligible errors)

$$G^{el}(R) = 8\epsilon a\left(\frac{kT}{e} \tanh\frac{e\psi_s^\infty}{4kT}\right)^2 e^{-\kappa R} \tag{6}$$

which corresponds to the so-called linear superposition approximation (Verwey & Overbeek, 1948), where ψ_s^∞ (i.e., ψ_s at $l = \infty$) is the root of

$$2 \sinh\frac{e\psi_{s\infty}}{2kT} = -\frac{4\pi e^2}{\epsilon kT\kappa S}\left[1 + Kn \exp\left(-\frac{e\psi_s^\infty}{kT}\right)\right]^{-1} \tag{7}$$

which is derived from eq 1, 3, and 4 with $l = \infty$.

The van der Waals interaction energy of two vesicles (which can be considered to be a shell of outer radius r and thickness h) at separation R is expressed (Ohsawa et al., 1981) as

$$G^A(R) = V(r,r,R + 2r) + V(r - h,r - h,R + 2r) - 2V(r - h,r,R + 2r) \tag{8}$$

where

$$V(r_1,r_2,d) = -\frac{A}{6}\left[\frac{2r_1r_2}{d^2 - (r_1 + r_2)^2} + \frac{2r_1r_2}{d^2 - (r_1 - r_2)^2} + \ln\frac{d^2 - (r_1 + r_2)^2}{d^2 - (r_1 - r_2)^2}\right] \tag{9}$$

is the van der Waals interaction energy of two solid spheres of radii r_1 and r_2 at distance d between their centers and A is the Hamaker constant.

Thus, the total interaction energy $G_t(R)$ of two vesicles is given by

$$G_t(R) = G^{el}(R) + G^A(R)$$

We have calculated $G_t(R)$ (Figure 7) as a function of R, r, and n with the following values: $T = 297$ K, $\epsilon = 79$, $h = 50$ Å, $S = 85$ Å^2 for a small vesicle ($2r = 300$ Å), and 70 Å^2 for two large vesicles of different sizes ($2r = 1500$ and 3000 Å) (Brouillette et al., 1982), $K = 0.7$ M^{-1} [the average value of those obtained by Nir et al. (1978) and Ohki & Kurland (1981)], and $A = 4 \times 10^{-14}$ erg (Gingell & Parsegian, 1972; Brooks et al., 1975; Requena & Haydon, 1975; Ohshima et al., 1982).

Registry No. Na, 7440-23-5; Li, 7439-93-2; K, 7440-09-7; Cs, 7440-46-2.

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Escherichia coli Single-Stranded DNA Binding Protein Is Mobile on DNA: ^{1}H NMR Study of Its Interaction with Oligo- and Polynucleotides†

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ABSTRACT: The interaction of the *Escherichia coli* single-stranded DNA binding protein (SSB) with oligo- and polynucleotides has been studied by 270-MHz ^{1}H NMR spectroscopy and fast kinetic techniques. $d(pT)_8$ and poly(dT) were used to study noncooperative and cooperative binding, respectively. The H6, H1′, and CH_3 resonances of $d(pT)_8$ are high-field shifted by less than 0.05 ppm, and H8 and H2 of poly(dA) are low-field shifted upon complexation. The protein resonances remain virtually unshifted. The small shifts upon complexation provide no evidence for extensive stacking interactions between the nucleotide bases and aromatic amino acid side chains of SSB. The $d(pT)_8$ and poly(dA) signals are broadened to about 30 Hz whereas the resonances of poly(dT) are broadened beyond detection upon stoichiometric complexation. Continuous broadening of all poly(dT) signals even at a 10-fold excess of poly(dT) indicates fast exchange of SSB between different binding sites. Dissociation and reassociation rates determined from stopped-flow experiments are too slow by at least 2 orders of magnitude to account for the experimental line widths. Therefore, we conclude that SSB translocates without dissociation from the DNA template. A model for the translocation is outlined. It is based on partial dissociation of octamer sections of poly(dT) from the complex with a rate constant as previously published for the dissociation of $d(pT)_8$ from SSB.

Single-stranded DNA binding proteins perform specific functions during the replication of DNA [for reviews, see Coleman & Oakley (1980), Kornberg (1980), Kowalczykowski et al. (1981), and Kornberg (1982)]. They bind preferentially to single-stranded DNA and much more weakly to double-stranded DNA, thereby destabilizing double-stranded DNA. This property facilitates the removal of secondary structures in the template (LaDuca et al., 1983). Furthermore, single-stranded DNA binding proteins promote the renaturation of DNA (Christiansen & Baldwin, 1977) and protect single-stranded DNA against attack of nucleases (Geider, 1978).

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