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Colloid Chemical Studies on the Aggregation of Phospholipid Vesicles with Metal Cations

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Aggregation behavior of phosphatidylethanolamine (PE) and Phosphatidylserine (PS) vesicles that have resulted with various metal cations, has been studied to determine their adsorption characteristics. The critical flocculation concentration of these cations for both vesicles was determined by two techniques, *i.e.*, by the kinetic method in which the rate of flocculation was measured turbidimetrically, and by the traditional static method. The results have been analysed by the usual stability concept in the colloid chemistry, and these analyses revealed that there are two mechanisms in the vesicle aggregation. One is the charge neutralization of the polar groups on the vesicle surface, which is induced by the adsorption of multivalent cations (Th^{4+}, La^{3+}), and the other is the interparticle binding (or the interparticle bridging) caused by the divalent cations (Ca^{2+}, Mg^{2+}).

The importance of the interaction of metal cations with natural membranes has been pointed out by many authors in connection with ion permeability through membranes,[1] membrane stability[2-4] and ion adsorption on the membranes,[5] *etc.* In order to obtain some insight into the mechanism of ionic interaction with biological membranes, several authors have investigated the surface potential of monolayers[6,7] made of phospholipids in various ionic environments. Bangham *et al.*[8] observed the different effects of various divalent cations on the surface potential of acidic phospholipid monolayers. Mclaughlin *et al.*[9] obtained the association constants of various divalent cations by measuring the conductance of phosphatidylserine membranes. Barton[10] determined the degree of divalent cation association with phosphatidylserine membranes by measuring the electrophoretic mobility of phospholipid vesicles.

Only a few colloid chemical studies on the aggregation of phospholipid vesicles have been reported. It has generally been observed that acidic phospholipid vesicles, such as phosphatidylserine and phosphatidylethanolamine vesicles, are very sensitive to multivalent cations;[11,12] the addition of relatively small amounts of cations can cause them to aggregate. In this study, the aggregation behavior of phosphatidylethanolamine and phosphatidylserine vesicles have been studied using various metal cations as coagulants and the experimental results have been analysed by the stability concept in the colloid chemistry.[13]

The concentration of the counter ions required to aggregate the colloidal dispersion is called the critical flocculation concentration(CFC) or flocculation value (C_{CFC}). The CFC depends on a number of factors, such as the nature of colloid particles, the surface charge, the double layer thickness of the particles, and the valence and nature of the counter ions. The procedures for measuring the CFC can be divided into (1) the kinetic method and (2) the static method. In the kinetic method, the initial rate of aggregation is measured turbidimetrically as a function of the concentration of the coagulation agent at which further increase of the coagulant produces no further speeding up of the aggregation process. For a better understanding of colloid stability, measurements in the period immediately after mixing were indispensable because these results are more suitable to interpretation in terms of the particle-particle interaction than those obtained by the static method.

In the static method, a row of glass tubes including the colloidal suspension is prepared, increasing amounts of the coagulant are added and the CFC is determined after waiting a few hours. This method has the drawback that the stability criterion is somewhat arbitrary, but the advantage is that, if properly done, a time scale can be used to attain adsorption equilibrium between the colloidal particles and the coagulation agent. The turbidimetric titration technique can be regarded as a static method, if the titrants are added slowly to attain the adsorption equilibrium.

In this work, two types of measurement on the CFC of the phospholipid vesicles have been undertaken, and the resulting data have been analysed systematically. From these analyses, it appeared that these two techniques give independently important knowledge on the interaction of metal cations with phospholipid membrane.

Experimental

Materials. Bovine brain phosphatidylethanolamine (PE) and phosphatidylserine(PS) were used as the phospholipids which were purchased from Sigma Chemical Co., Ltd (U. S. A.). Other reagents used were of an analytical grade. All the solutions of these materials were made with deionized and doubly distilled water.

Vesicle Preparation. PE and PS vesicles were prepared by the sonication method at 4°C without using any buffer to reduce the spurious effects of the included buffer ions.[14] After the removal of the undispersed materials by the centrifugation (26000g, 1h), the dispersion was filtered through a membrane filter (0.1 μm pore size).

Turbidimetric Titration. Turbidimetric titration was carried out at 25°C under a nitrogen atmosphere using a Hirama automatic recording titrator (Tokyo Japan). The

transmittance (or turbidity) of the vesicle dispersion (30ml, 1—8×10^{-2}g/dl) was measured at the wavelength of 517nm. The titrant solution (0.01—0.3 mol dm^{-3} salt solution) was added very slowly (0.01ml/min) to the vesicle dispersion to attain the adsorption equilibrium between the vesicle surface and the titrant ions. As the titration proceeds, the dispersion begins to become turbid, and the transmittance reaches a minimum at a certain titrant volume (V_{min}) from which we have calculated the CFC on each condition.[12]

Measurements of Aggregation Rate. A Union stopped-flow spectrophotometer (Tokyo, Japan) with a 1cm path length cell was used in the measurements. In this apparatus, the liquids to be mixed are contained in two syringes. By using a pressure actuator, equal volumes (1.5ml) of each solution are reproducibly mixed within a very short time (10ms). The turbidity of the mixture can be recorded on an attached recorder and the initial slope of the optical density (A) *vs.* time (t) curves can be measured. The CFC-value can be determined by plotting the logarithm of the reciprocal slope (log dt/dA) against log c, where c is the coagulant concentration. The measurements are carried out at room temperature and at a wavelength of 517nm.

Electrophoresis. The electrophoretic mobilities of large vesicles were determined at various pH values in an aqueous solution at 25°C. The measurements were performed in a Rank brother micro-electrophoretic apparatus (MK-2) using a rectangular glass cell.

Adsorption Experiments. Ten ml of vesicle dispersion (0.05g/dl) were mixed with the various salt solutions containing different ion concentrations and left standing for one night at 25°C. Only the vesicle particles were separated from the medium by ultrafiltration (Millipore PTGC) and the concentration of metal cations remaining in the filtrate as well as in the initial solution was measured analytically by means of the Inductively Coupled Argon Plasma Method (Jarrell-Ash 975). From the difference in the concentration before and after the adsorption process, the amounts of adsorption per unit of the vesicle weight (Ap) were calculated.

Membrane Potential Measurements. Monolayers of phospholipids were prepared by spreading chloroform–methanol (3:1) solution of phospholipid on the surface of subphase solution at the neutral pH in 80 ml Teflon trough. After a concentrated salt solution containing the metal cations was added to the subphase solution, the subphase solution was stirred for more than 30 min with a glass-sealed needle (5 mm length) by means of a magnetic stirrer. Thereafter, the surface potentials of the monolayer were measured as a function of salt concentration with a vibrating electrode against an Ag-AgCl reference electrode. All the monolayers used in these experiments have a packing density of 1/60(molecule/Å).

Results and Discussion

Static Method. In Fig. 1, some typical turbidimetric titration curves of PS vesicles with an added $ThCl_4$ aqueous solution are shown as a result of changing the vesicle concentration (C_p). As the titration proceeds, the vesicle dispersion begins to become turbid and the transmittance reaches a minimum at a certain titrant volume (V_{min}), from which we can calculate the CFC for each condition by connecting for the total volume of the sample solution and the added titrant. The V_{min} (or CFC)-value is much dependent on the C_p and the plot of the V_{min} (or CFC) against the C_p shows a straight line passing through the origin as shown in Fig. 2. This indicates that PS vesicles are quantitatively coagulated with Th^{4+} ions. We previously found that phosphatidylcholine (PC) vesicles are also coagulated quantitatively with trimethylammonium glycol-chitosan iodide (TAGC) and concluded that the vesicle coagulation with TAGC is based on a new salt-linkage formation between the phosphate group of PC and the trimethylammonium group in TAGC.[15] From the colloid chemical aspect, this phenomena can be explained as follows: The negative charge on the vesicle particle is neutralized by the adsorption of Th^{4+} ions, and the vesicle particles with zero-charge start to coagulate by the London-Vander Waals attraction forces.[13] In Fig. 2, a relation between the CFC and the C_p of PE vesicles is also plotted. A comparison of these two curves, reveals that the CFC for PS vesicles is always higher than the value of PE vesicles at a constant C_p. This difference will be based on the molecular structure of these phospholipids; the PS molecule has two species of the acidic polar groups (carboxyl and phosphate groups), and the PE molecule has only one group (phosphate group). Therefore, to complete the charge neutralization of the PS molecule, twice the number of cations will be necessary compared to

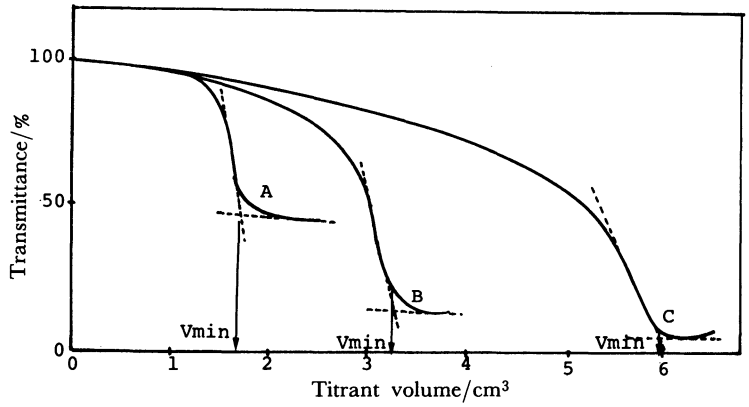

Fig. 1. Typical turbidimetric titration curves of PS vesicles with $ThCl_4$ solution. A: C_p=0.014g/dl, B: C_p=0.0423g/dl, C: C_p=0.0750g/dl.

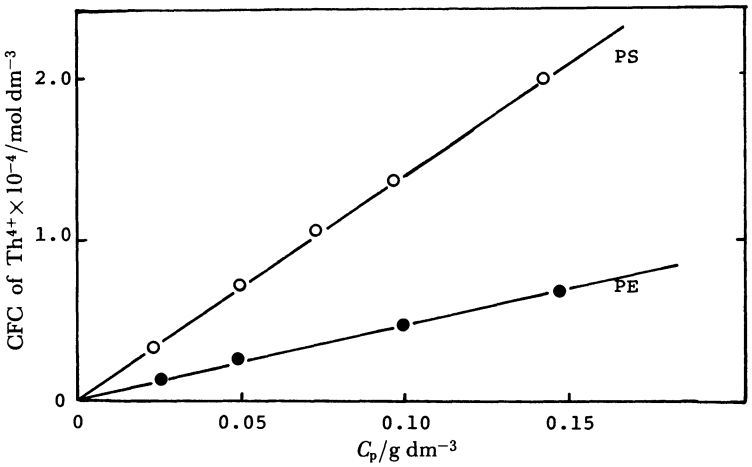

Fig. 2. Relations between the CFC of $ThCl_4$ and the C_p of PS and PE vesicles (pH 4.0).

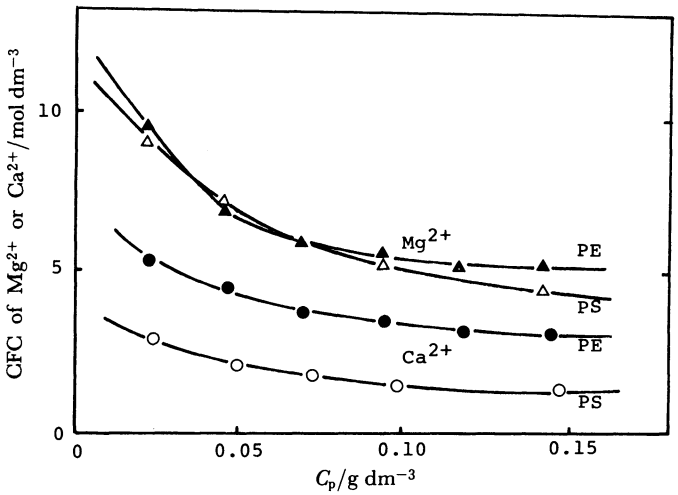

Fig. 3. Relations between the CFC of divalent cations and the C_p of PS and PE vesicles (pH 4.0).

the PE molecule. The tendency of Fig. 2 accords approximately with this assumption, and indicates again that the vesicle coagulation in these systems will be based on the charge neutralization of polar groups on the vesicle surface.

In Fig. 3, the relations between the CFC of divalent cations and the C_p of PE and PS vesicles are plotted. In contrast to Fig. 2, all the curves are decreasing with increasing the C_p. It means that a vesicle suspension with a high C_p can be coagulated by a lower salt solution than the suspension with a low C_p. Furthermore, the relation between the CFC-values of PE and PS vesicles is the reverse of the relation in Fig. 2. These results will oblige us to adopt a different coagulation mechanism from one in Fig. 2. The new mechanism will be an interparticle binding (or an interparticle bridging) caused by the addition of a divalent cation. Existense of the new mechanism is also confirmed by observation of the vesicle aggregates formed by the addition of the various metal cations, *i.e.*, the aggregates formed by Th^{4+} are compact and relatively small flock, while the aggregates formed by Ca^{2+} are large and loose in structure. The existence of two such aggregation mechanisms will be based on the different binding affinity of the added metal cations. Figure 4 shows the initial slope of adsorption isotherms of various metal cations to PE vesicles. The

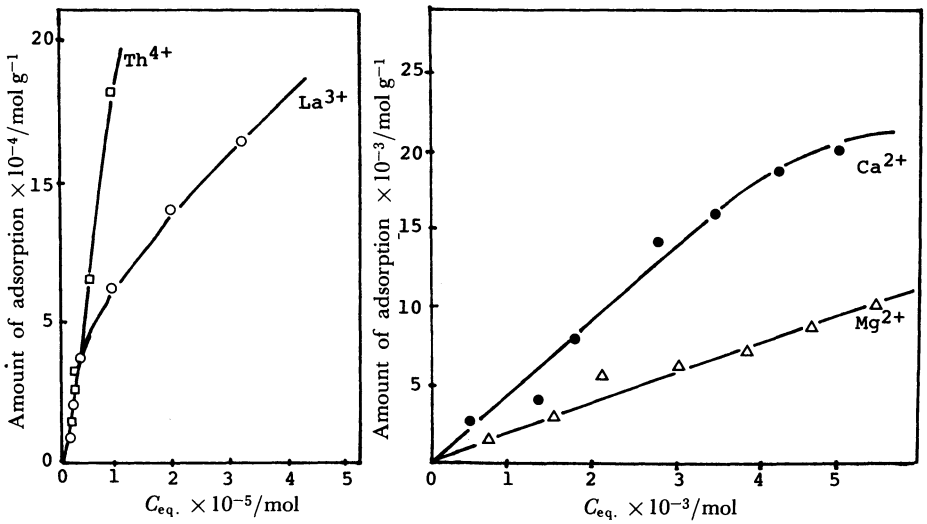

Fig. 4. Initial slope of adsorption isotherms of various metal cations to PE vesicles.

affinity is much dependent on the valence of the cation and especially Th^{4+} and La^{3+} ions show high affinities. From a comparison of Fig. 4 with Fig. 2(or Fig. 3), it is apparent that Th^{4+} or La^{3+} ion with a high adsorption affinity causes the vesicle aggregation due to the charge neutralization, and Ca^{2+} or Mg^{2+} ion with a moderate affinity brings to the aggregation based on the interparticle bridging. In the latter mechanism, the aggregation efficiency will increase by increasing the particle concentration, which is the same tendency with the result found in Fig. 3. Furthermore, this analysis explains reasonably the difference of coagulation behavior of Ca^{2+} and Mg^{2+} ions (see Fig. 3), *i.e.*, Ca^{2+} will make a bridge in higher efficiency than Mg^{2+} by its high adsorption affinity and such a tendency will appear definitely on PS vesicles with two acidic groups. Thus, Ca^{2+} ion has a lower CFC than Mg^{2+} ion, especially for PS vesicles.

Kinetic Method. Figure 5 shows some typical curves of optical density (A) *vs.* time (t), obtained by adding $CaCl_2$ aq solution to PE vesicles. In this figure, the salt concentrations indicated are the final concentrations after mixing by the stopped-flow method. It is clear from these curves that the initial portions are sufficiently linear in agreement with theoretical expectations.[16] The deviation from linearity is due to the non validity of the assumption that the scattering from aggregates is the same as that from spherical particles of the same total weight. By increasing the salt concentrations, the initial slope increases until finally a maximum region is attained where the coagulation rate becomes independent over the wide range of salt concentrations. The CFC is easily determined by plotting the logarithm of the reciprocal slope at the initial time (log dt/dA) which is proportional to the theoretical stability ratio,[13] against log c, where c is the coagulant concentration. In Fig. 6, the plots of log dt/dA *vs.* log c are given for PE and PS vesicles using $CaCl_2$ as the coagulant. The curves obtained are not of the forms predicted by the simple stability theory. They are linearly decreasing in the slow coagulation range and, after staying at a minimum value, they increase again in the high concentration region of $CaCl_2$. Each inflection point in the slow coagulation range is nearly equal to the CFC obtained from the turbidimetric titration.

The occurrence of the minimum stability ratio can be explained in terms of the interparticle bridging of Ca^{2+} ion, because the particle coagulation due to the bridging is a bimolecular process, the rate must be proportional to the product, $\theta(1-\theta)$, of the fraction of covered (θ) and uncovered ($1-\theta$) with adsorbed Ca^{2+} ions.[17] As may be seen in Fig. 6, the experimental points for both vesicles lie within experimental error on a parabola, corroborating the bimolecular coagulation,[18] *i.e.*, the rapid coagulation rate in the middle concentration region, changes again to the slow rate to lose the fraction of uncovered particle. The difference in

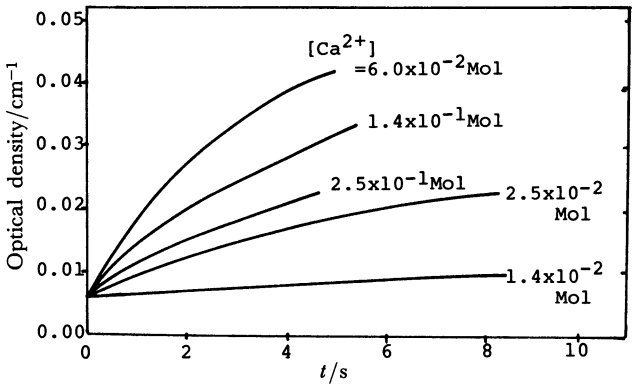

Fig. 5. Optical density (A) *vs.* mixing time curves for PE vesicles.

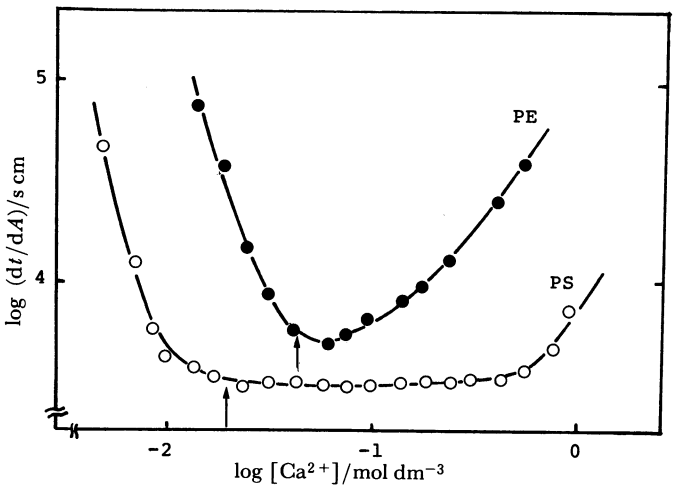

Fig. 6. Plots of logdt/dA *vs.* log[Ca^{2+}] for PE and PS vesicles (pH 4.0)

the stability curves of PE and PS vesicles in Fig. 6, may be explained by the different adsorption capacities of Ca^{2+} for both vesicles. In other words, the number of adsorption sites on PS vesicles is more than the site on PE vesicles. This is testified by the measurements of membrane potential for PE and PS monolayers. Figure 7 shows the membrane potential change (ΔV) of both monolayers with respect to the $CaCl_2$ concentration. Increasing the $CaCl_2$ concentration induces a gradual increase in ΔV and above 5×10^{-2} mol, the ΔV attains respective plateaus which will be proportional to the number of adsorption sites of Ca^{2+}. As may be seen from Fig. 7, the number of sites on the PS membrane is about double number of sites on the PE monolayer, which is an essential reason why PE and PS vesicles would take different aggregation behavior. Further, the detailed analysis between the experimental points of stability and the theoretical curve, will be discussed in the next paper of this series.

In Fig. 8, the stability curves for PE and PS vesicles using $LaCl_3$ are given. The curves of stability, especially for PS vesicles, have a form predicted from the simple stability theory,[19] *i.e.*, it is linear in the slow coagulation range and becomes parallel to the abscissa in the rapid coagulation range. However, the stabil-

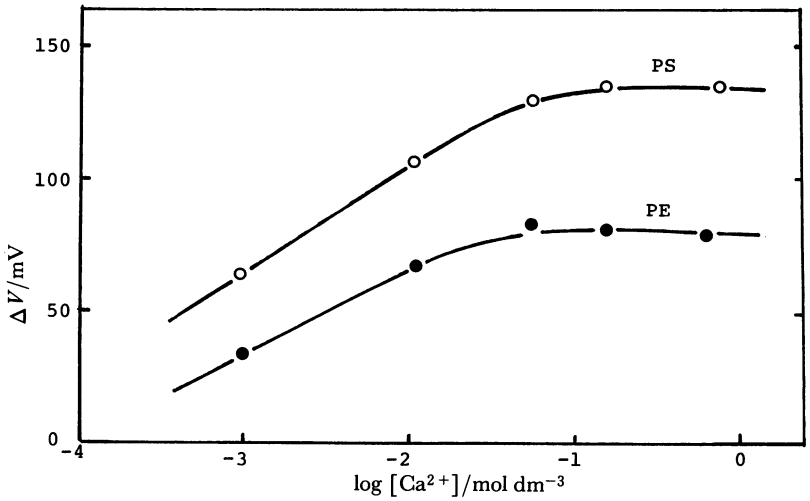

Fig. 7. Membrane potential change (ΔV) of PS and PE monolayers with $CaCl_2$ concentrations (pH 6—pH 8, 25°C).

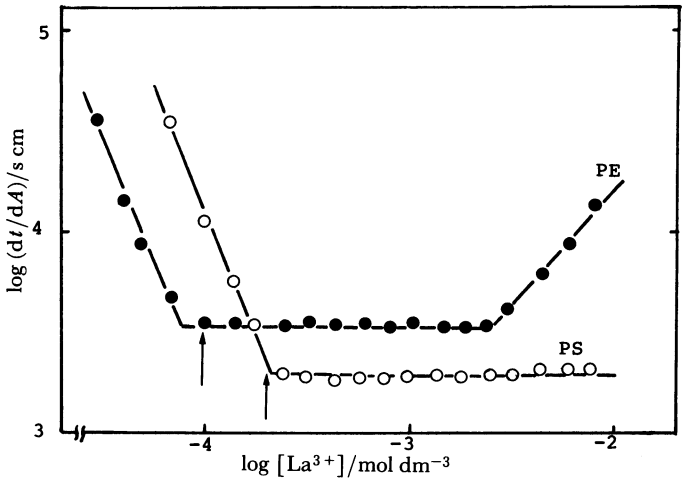

Fig. 8. Plots of $\log dt/dA$ *vs.* $\log[La^{3+}]$ for PE and PS vesicles (pH 4.0).

ity curve for PE vesicles has a slightly different form from the curve for PS vesicles, and is scarcely increased again in the high concentration region of the salt. As the vesicle coagulation with $LaCl_3$ is based on charge neutralization of polar groups on the surface, this difference between PE and PS vesicles in Fig. 8, must be explained in terms of electrostatic interaction between the particles. Figure 9 shows of zeta-potential *vs.* log molar concentration of $LaCl_3$ for PE and PS vesicles. As the concentration of $LaCl_3$ increases, the zeta-potential gradually decreases with the different slope in PE and PS vesicles; PS vesicles kept slightly negative values until the concentration of 1×10^{-2}mol $LaCl_3$, while, PE vesicles approach to zero and finally become to positive zeta-potential at the same $LaCl_3$ concentration. These electrophoretic results for PE vesicles suggest that the stability behavior in Fig. 8 is due to the reversal of vesicle charge. On the other hand, PS vesicles hardly reverse their electric sign and continue a low stability value over the wide range of $LaCl_3$ concentrations.

In conclusion, it may be said that the usual coagulation technique in the colloid chemistry is the best suitable for studying the properties of the membrane surface, and this gives important knowledge on the conformation and functionality of the polar head groups on the surface of the membrane.

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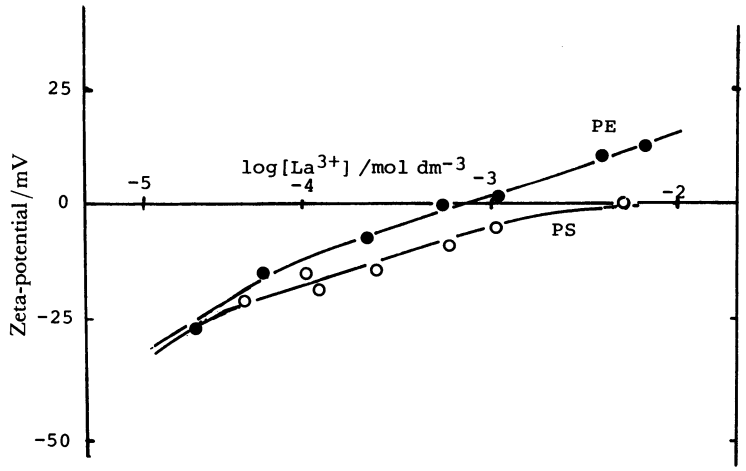

Fig. 9. Zeta-potential *vs.* $\log[La^{3+}]$ curves of PE and PS vesicles (pH 4.0, 25°C).

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