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BINDING AND AGGREGATE FORMATION OF PHOSPHOLIPID LIPOSOMES CONTAINING PHOSPHATIDIC ACID WITH OVALBUMIN

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SUMMARY

The interaction of ovalbumin with the phospholipid liposomes formed by ultrasonic vibration was investigated by means of the aggregate formation using low-speed centrifugation or filtration. Phospholipids were precipitated by interaction of the liposomes formed from a commercial phosphatidylcholine preparation with ovalbumin in acidic media of pH 3.5–2.0, but not in the media above pH 4.0 or below pH 1.5. The maximum amount of phospholipids was found in the precipitate obtained at pH 2.5.

Among the liposomes formed from pure phospholipids (*i.e.* phosphatidylcholine, phosphatidylethanolamine, or phosphatidic acid) phosphatidic acid liposomes alone formed an aggregate by binding with the protein at pH 2.5, while other two phospholipids did not bind with the protein. When liposomes consisting of a mixture of phospholipids were used, only mixed phospholipid liposomes containing phosphatidic acid bound with ovalbumin at pH 2.5. It may be concluded that the component in the commercial phosphatidylcholine responsible for interaction with ovalbumin is phosphatidic acid.

Aggregate formation between the dispersions from pure phosphatidic acid and ovalbumin was anomalous. This was prevented by the addition of an excess of either the protein or the lipid. The amount of phosphatidic acid bound to ovalbumin was approximately a minimum of 14 and a maximum of 77 moles of the lipid per mole of the protein.

Interaction of the liposomes from the commercial phosphatidylcholine with ovalbumin was inhibited by increased ionic strength or by the presence of a bivalent cation, Ca^{2+}. Phospholipids from the precipitate were found in the supernatant by sonication at pH 2.5 or by extraction with a buffer of pH 6.0, but only slightly by the extraction with buffer of pH 2.5.

It is thought that the results presented here are best explained by primary electrostatic interaction between the protein and phosphatidic acid, followed by other secondary weak interactions leading to the observed aggregate formation.

INTRODUCTION

It has been extensively shown that phospholipid dispersions in excess water exist as liposomes having closed multilamellar structure, which are highly im-

permeable to ions[1,2]. These multilamellar preparations have been used to examine the molecular architecture obtained after the binding of soluble basic proteins, such as cytochrome *c*, by X-ray diffraction techniques[3–5], electron microscopy[6], and spin labeling[7]. From a functional aspect, these liposomes have also been used for studying the permeability effect due to interaction of proteins with phospholipids[8,9].

Sonic oscillation of phospholipid dispersions produces closed unilamellar liposomes[3,10]. It was shown that the liposomes bound with some basic proteins are accompanied with changes in Na^+ permeability[11]. The work reported here is an investigation on the interactions of ovalbumin, an acidic glycoprotein, with phospholipid liposomes formed by ultrasonic vibration. It was found that, under certain conditions, this protein produces phospholipid liposomes from a commercial phosphatidylcholine preparation, phosphatidic acid, or mixed phospholipids containing phosphatidic acid to precipitate with their aggregate formation.

MATERIALS AND METHODS

Ovalbumin (5 times crystallized) was obtained from Nutritional Biochemicals Corp. The protein showed a somewhat broad, single band on disc electropherogram, indicating no significant contamination with other proteins. A commercial egg yolk phosphatidylcholine preparation used in this experiment was purchased from E. Merck AG. The preparation contained, except for phosphatidylcholine, a small amount of phosphatidylethanolamine and phosphatidic acid and traces of lysophosphatidylcholine and sphingomyelin, but not free fatty acid, by thin-layer chromatography on silica gel G (E. Merck AG.) in chloroform–methanol–acetic acid–water (25:15:4:2, by vol.). The phosphatidic acid content of the preparation was approx. 7.3%, as calculated from titration at pH 6.63, while the phosphatidylethanolamine content was approx. 4.8% by computation from ethanolamine content by an amino acid analyzer after hydrolysis in 6 M HCl at 110 °C for 24 h, assuming the mol. wt of phospholipids to be 900. Pure phosphatidylcholine was prepared from fresh hen's egg by the method of Papahadjopoulos and Miller[3]. Phosphatidic acid was prepared from this phosphatidylcholine by digestion with phospholipase D from carrot[12]. Phosphatidylethanolamine was also prepared from the eggs by the method of Lea *et al.*[13]. The purity of these three phospholipids was checked by thin-layer chromatography. All other reagents were analytical reagent grade and used without further purification.

The buffers used in this study were prepared by as follows: adjusting the pH of a mixture of 0.04 M phosphoric acid, acetic acid and boric acid to the desired pH with 0.2 M NaOH (first buffer); by the addition of 0.1 M HCl to a mixture of 0.05 M Tris and sodium acetate (second buffer). For buffers with a pH below 1.5, 1 M HCl was added to the second buffer (pH 2.0).

Absorbance was determined with a Shimadzu Multipurpose recording spectrophotometer, MPS-50L, and turbidity with a Hitachi recording spectrophotometer, EPS-3. Sonication was carried out with a Tominaga supersonic vibrator, UR-150P, and centrifugation with a Tominaga swing-type centrifuge, CD-50S. pH was measured by a Hitachi–Horiba pH meter, M-5.

Preparation of phospholipid liposomes

Phospholipid suspended in distilled water was dispersed immediately before use by ultrasonic oscillation (20 kcycles, 5 A) in an ice bath until a homogeneous dispersion was obtained, except in the case of phosphatidic acid and commercial phosphatidylcholine. The latter two dispersions were stored in a refrigerator and used without further treatment. In the case of liposomes from mixed phospholipids, ether solutions of each phospholipid were mixed and sonicated by the same method as above, after ether was evaporated under vacuum.

Measurement of interaction of phospholipid with ovalbumin

A mixture of the known amounts of phospholipid, ovalbumin, and buffer of the indicated pH in a total volume of 2.0 ml was incubated at 37 °C for 1 h. The mixture was centrifuged at 3000 rev./min (4000 × *g*) for 20 min at room temperature. In the case of the dispersions from phosphatidic acid alone, the mixture was filtered through Toyo filter paper No. 2 (5.5 cm in diameter) because of flocculence formation.

Analyses for phospholipid and protein

Phospholipid was determined by comparing the amount of the lipid originally used with that recovered in the aggregate by the hydroxamate method of Rapport and Alonzo[14], after drying over P_2O_5 under a reduced pressure. Protein in the supernatant was determined by the method of Lowry *et al.*[15]. Protein in the aggregate was calculated by subtracting the amount remaining in supernatant from that originally used.

RESULTS

Liposomes prepared from a commercial phosphatidylcholine by ultrasonic vibration were very stable in water and no change in turbidity was observed for 1 month in a refrigerator. The liposomes were found to give a single, sharp, symmetrical peak near the void volume on Sephadex G-150 gel filtration. When the liposomes were incubated at 37 °C for 1 h in the second buffer between pH 1.5 and 10.0 with or without 10^{-2} M $CaCl_2$, no precipitation was found after centrifugation. In the case of liposomes made by mechanical shaking, however, a part of the lipid was recovered in the precipitate after this low-speed centrifugation. This finding may reflect a difference in lamellar structure between these liposomes prepared by the two distinct methods.

Interaction of ovalbumin with the liposomes formed by sonic irradiation was examined as a function of pH. By addition of the same amount of the protein to the system, no precipitation of the phospholipids was observed in the solutions above pH 5.5. Moreover, when a mixture of the liposomes and ovalbumin was filtered on a Sephadex G-150 column at pH 7.5 or 9.5, the two components were completely separated from each other. The phospholipids were eluted near the void volume, as well as phospholipids alone, while ovalbumin was eluted in the same fractions as the protein alone. These data indicate that ovalbumin does not interact with the liposomes in solutions near and above neutral pH values. As can be seen in Fig. 1, however, phospholipids were precipitated from acidic media in the presence of ovalbumin. A significant amount of phospholipids was found in the precipitate

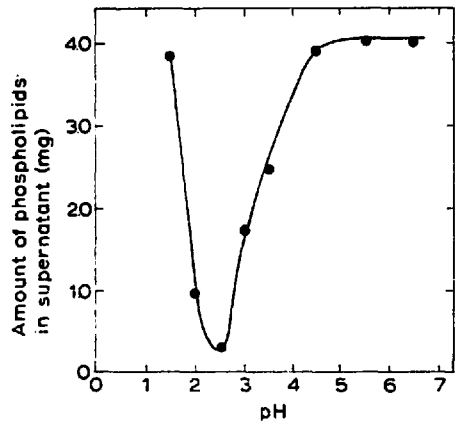

Fig. 1. Aggregate formation from a commercial phosphatidylcholine liposomes with ovalbumin. A mixture of ovalbumin (4.0 mg) and the liposomes (4.0 mg) in a total volume of 4.0 ml of the second buffer, described in the text, was centrifuged at 3000 rev./min (4000 × *g*) for 20 min at room temperature after incubation at 37 °C for 1 h. Phospholipids in the precipitate were determined by the hydroxamate method[14] and the difference between added and recovered lipids was plotted as the lipid remaining in the supernatant.

obtained at pH values between 2.0 and 3.5. The protein was not detected in the precipitate under the conditions used. In this experiment, in which the second buffer containing Tris was employed, the color development with protein was considerably low and showed incorrect linearity to its concentration. Thus, a little change in the amount of protein was not correctly measured. In another experiment, in which the first buffer was used, approx. 8% of the protein was recovered in the precipitate obtained at pH 2.5, accompanied with the recovery of 90% of phospholipids. This fact shows that precipitation of both phospholipids and ovalbumin was based on the aggregate formation by binding of the protein to the liposomes.

Since the commercial phosphatidylcholine preparation contains a small amount of phosphatidylethanolamine and phosphatidic acid as major contaminants, one of the questions to be solved with regard to the mechanism of lipid-protein interactions is which of the phospholipid components in the preparation participates in the binding with the protein. The individual three phospholipid liposomes in water were formed from pure phosphatidic acid, phosphatidylethanolamine and phosphatidylcholine by sonic oscillation. These liposomes were diluted 20-fold with the first buffer (pH 2.5) and centrifuged at 3000 rev./min for 20 min after incubation at 37 °C for 1 h. The results are shown in Table I. Phosphatidic acid dispersions did not form any visible aggregate by such a treatment, but a considerable amount of phosphatidylethanolamine or phosphatidylcholine was found in the precipitate. The amount precipitated was larger from phosphatidylethanolamine than from phosphatidylcholine liposomes. Moreover, phosphatidic acid dispersions were very stable and could be stored at least for a month in a refrigerator without any formation of an aggregate. Phosphatidylcholine liposomes having a high viscosity formed visible flocculence after standing for a few hours at room temperature, while turbidity of phosphatidylethanolamine liposomes increased progressively and after sonication an aggregate was gradually formed. When phosphatidylethanolamine liposomes diluted with the first buffer were sonicated once before incubation, the amount of phosphatidylethanolamine precipitated was roughly equal to that of phosphatidylcholine lipo-

TABLE I

INTERACTION OF OVALBUMIN WITH THE LIPOSOMES OF PURE PHOSPHOLIPIDS

A mixture of 1 mg of ovalbumin and 1 mg of phospholipid in 2.0 ml of the first buffer (pH 2.5) was treated by the method described in the legend to Fig. 1. Ovalbumin remaining in the supernatant was determined and the amount bound was calculated by difference.

Liposomes	*Amounts bound (μg)*		
	Without protein	*With protein*	
	Lipid	*Lipid*	*Protein*
Phosphatidic acid*	0	661	993
Phosphatidylcholine	397	375	0
Phosphatidylethanolamine	691	677	0
Phosphatidylethanolamine**	348	96	0

* Filtered by the method described in Materials and Methods.

** Liposomes were also subjected to a 1-min sonication after dilution with the buffer and addition of the protein.

somes without further sonication. A small amount of phosphatidylcholine was found in the precipitate centrifuged without incubation. In the case of phosphatidylethanolamine liposomes, almost the same amount of the lipid was precipitated with or without incubation. These results indicate that phosphatidylethanolamine liposomes have a greater tendency to aggregate rapidly than phosphatidylcholine ones, in particular, at a high concentration of the lipid.

By addition of the same amount of ovalbumin to the system, formation of an aggregate from phosphatidic acid dispersions was observed, although no aggregation occurred in the absence of the protein. Both the protein and phospholipid were found in the aggregate, in which almost all of the protein and 66.1% of phosphatidic acid added were recovered. On the other hand, ovalbumin was not detected in the precipitate obtained from phosphatidylethanolamine or phosphatidylcholine liposomes. The protein, moreover, did not show any definite effect on phosphatidylethanolamine or phosphatidylcholine precipitation, except for a small decrease in the amount of the lipids precipitated. This inhibitory effect was clear on the precipitation of phosphatidylethanolamine liposomes which were once sonicated in the presence of the protein. Only 9% of the phosphatidylethanolamine added was found in the aggregate by the addition of ovalbumin, while 32% of the lipid was found in its absence. Although it is not clear at present, why the aggregation of phosphatidylethanolamine was prevented in the presence of the protein, it is plausible that the increase of viscosity of the medium by the addition of ovalbumin may have stabilized phosphatidylethanolamine liposomes. The results presented in Table I indicate that ovalbumin binds with phosphatidic acid but not with phosphatidylethanolamine or phosphatidylcholine under the conditions used. At pH 2.5, the ovalbumin molecule was present as a net positively charged species, while phosphatidic acid was present as negatively charged particles. Thus, the protein may, at first, bind with the particles of phosphatidic acid by electrostatic interaction and then form the aggregate by secondary unknown factor(s). A reason that the aggregate formed did not completely precipitate by centrifugation may be due to low density of phosphatidic acid particles.

The binding of ovalbumin with phosphatidic acid dispersions was further studied. When the amount of phosphatidic acid was changed with increments of 0.1 mg to a constant value of 1 mg of the protein, the amount of the protein bound increased linearly with the concentration of phosphatidic acid over the range of 0.2 to 0.8 mg (Fig. 2). Almost all of the protein was found in the aggregate obtained at values above 0.9 mg of phosphatidic acid. By the use of one-tenth the amount of phosphatidic acid compared to the protein, the filtrate was turbid and a small amount of ovalbumin was recovered in the residue. This finding infers that the aggregation after interaction of ovalbumin with phosphatidic acid is suppressed by the presence of an excess of the protein. A nearly constant amount of phosphatidic acid was found in the aggregate formed over the range of 0.2–0.7 mg of the lipid, accompanied with an increase in the amount of the protein bound. Above a value 0.8 mg of phosphatidic acid added, the aggregate, which was separable by filtration but not by centrifugation, contained an increased amount of phosphatidic acid. This observation indicates that the amount of phosphatidic acid bound to the protein increased markedly in the presence of the lipid in amounts nearly equal to those of ovalbumin. A maximum ratio of ovalbumin to phosphatidic acid in the aggregate was obtained from a mixture of 1 mg of the protein and 0.7 mg of the lipid. In this case, 1 mg of ovalbumin was bound to approx. 0.25 mg of phosphatidic acid, that is, 14 moles of phosphatidic acid per mole of the protein, assuming the mol. wts of ovalbumin and phosphatidic acid to be 46000 and 810, respectively.

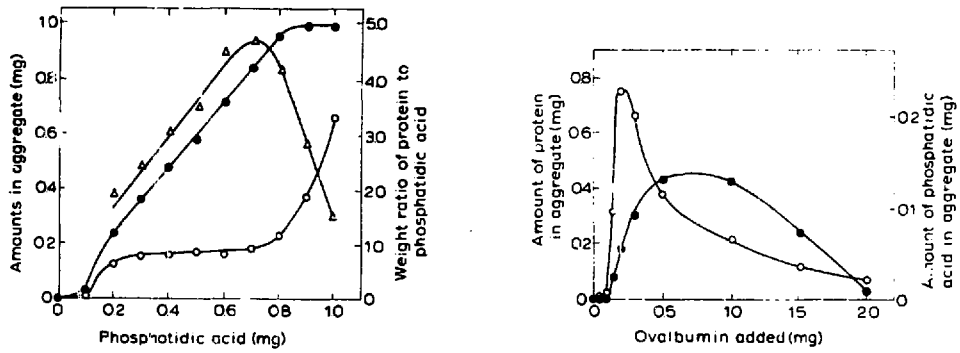

Fig. 2. Effect of phosphatidic acid concentration on its aggregate formation with ovalbumin. Mixtures of ovalbumin (1.0 mg) and phosphatidic acid dispersions in 2.0 ml of the first buffer (pH 2.5) were filtered through a filter paper after incubation for 1 h at 37 °C. The protein remaining in the filtrate was determined and the amount of ovalbumin bound to lipid was calculated from the difference. Phosphatidic acid in the residue was extracted 3 times with 3 ml each of a mixture of chloroform and methanol (2:1, v/v). After evaporation of solvent under vacuum, the lipid was determined by the hydroxamate method. ●—●, protein; ○—○, phospholipid; △—△, ratio.

Fig. 3. Effect of ovalbumin concentration on its aggregate formation with phosphatidic acid dispersions. Mixtures of phosphatidic acid dispersion (0.3 mg) and ovalbumin in 2.0 ml of the first buffer (pH 2.5) were treated as described in the legend to Fig. 2. ●—●, protein; ○—○, phospholipid.

When the amount of ovalbumin added was changed, the maximum amount of phosphatidic acid in the aggregate was obtained from a mixture consisting of 0.3 mg of phosphatidic acid and 0.2 mg of the protein (Fig. 3). A maximum ratio of phosphatidic acid to ovalbumin in the aggregates formed was also found in this aggregate, in which 1 mole of the protein bound to approx. 77 moles of phosphatidic

acid. In the presence of an excess of either ovalbumin or phosphatidic acid, each mixture was turbid after centrifugation. The turbidity in the supernatant was not removed by filtration. It appears from the data presented in Figs 2 and 3 that formation of a mass of aggregate, large enough for separating by centrifugation or filtration, is inhibited at a concentration of phosphatidic acid below one-tenth or above 3-fold that of the protein.

TABLE II

BINDING OF OVALBUMIN TO THE MIXED PHOSPHOLIPID LIPOSOMES

The conditions used were almost same as those described in the legend to Table I.

Liposomes	*Amounts bound (μg)*		
	Without protein	*With protein*	
	Lipid	*Lipid*	*Protein*
5% phosphatidic acid in phosphatidylcholine	75	493	102
5% phosphatidylethanolamine in phosphatidylcholine	192	96	0
5% phosphatidic acid and 5% phosphatidylethanolamine in phosphatidylcholine	22	595	110
Phosphatidylcholine + 10% phosphatidic acid in phosphatidylcholine*	31	385	117
Phosphatidylcholine + 10% phosphatidylethanolamine in phosphatidylcholine*	73	47	0
10% phosphatidic acid in phosphatidylcholine + 10% phosphatidylethanolamine in phosphatidylcholine*	35	367	120

* A mixture of each of the liposomes (1:1).

The result of the interaction of liposomes, formed from mixed phospholipids, with ovalbumin is presented in Table II. As expected from the data in Table I, in which phosphatidylethanolamine or phosphatidylcholine liposomes did not bind with the protein, the mixed phospholipid liposomes consisting of a mixture of phosphatidylethanolamine and phosphatidylcholine also did not bind with ovalbumin. Ovalbumin was detected only in the precipitate obtained from the mixed phospholipid liposomes containing phosphatidic acid. The amount of ovalbumin in these aggregates was nearly equal in the systems containing the same amount of phosphatidic acid and it was approx. 110 μg. Though the amount of ovalbumin added was 20 times that of phosphatidic acid in the mixed phospholipid liposomes, a part of these liposomes precipitated with aggregate formation due to the binding of ovalbumin with these liposomes. Compared with the dispersions from phosphatidic acid alone, aggregate formation from the mixed phospholipid liposomes containing phosphatidic acid was not prevented by the excess protein remaining after interaction. In the absence of ovalbumin only 7.5% of the phospholipids were recovered in the precipitate from the mixed phospholipid liposomes consisting of a mixture of phosphatidic acid and phosphatidylcholine, compared to approx. 40% precipitation from ones formed from phosphatidylcholine alone. Because of no precipitation from single phosphatidic acid dispersions, the difference may be due to suppression of the aggregation of phosphatidylcholine with phosphatidic acid.

From a mere mixture of phosphatidylcholine liposomes and mixed phospholipid ones from phosphatidic acid in phosphatidylcholine, 3.1% of the phospholipids precipitated. This result shows that the liposomes containing phosphatidic acid also stabilize single phosphatidylcholine liposomes having a tendency to form an aggregate. Similar results were also observed on mixed phospholipid liposomes containing phosphatidylethanolamine. Since phosphatidylethanolamine liposomes alone were less stable than phosphatidylcholine ones alone, it is still unknown why a small amount of phosphatidylethanolamine contained in the mixed phospholipid liposomes contributes to their stabilization. A suppression effect of ovalbumin was also observed on the mixed phospholipid liposomes consisting of phosphatidylethanolamine and phosphatidylcholine.

The results when the phosphatidic acid content of the mixed phospholipid liposomes is varied are presented in Fig. 4, which shows that precipitation of the phospholipids did not occur above 10% of phosphatidic acid in the liposomes in the absence of ovalbumin. By addition of the protein, roughly an equal amount of phospholipids was recovered in the precipitate formed, regardless of the phosphatidic acid content of the liposomes. The amount of ovalbumin precipitated increased progressively in proportion to the phosphatidic acid content of the mixed phospholipids liposomes. It should be noted that, as judged from the amount of phospholipids precipitated, phosphatidic acid alone was not present in the aggregate from the mixed phospholipid liposomes by binding with the protein. It seems from the data presented above that the stability of the liposomes from commercial phosphatidylcholine is mainly due to phosphatidic acid contained in the preparation as a contaminant. It is also strongly suggested that the principle responsible for the interaction with ovalbumin is phosphatidic acid in the commercial phosphatidylcholine liposomes.

To solve further the question of whether electrostatic interaction is the primary

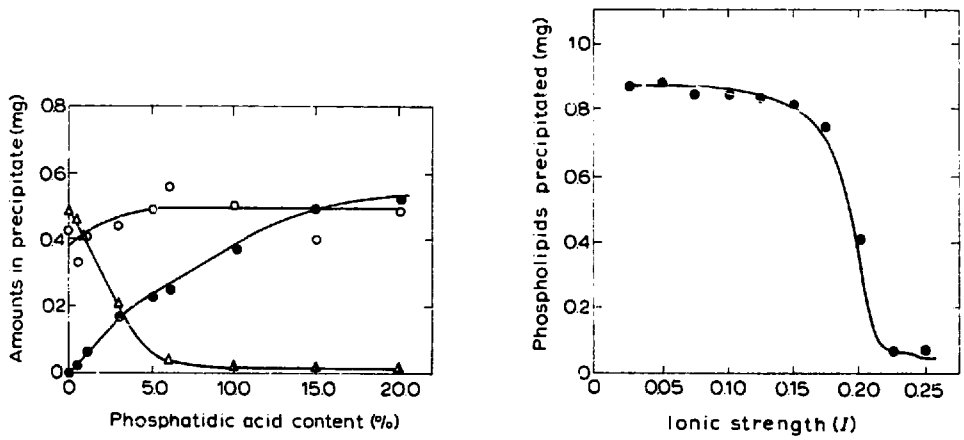

Fig. 4. Dependence of ovalbumin–phosphatidylcholine liposomes binding on phosphatidic acid. Phosphatidylcholine liposomes containing increasing amounts of phosphatidic acid were mixed with ovalbumin and treated as described in the legend to Table I. ●—●, protein; ○—○, phospholipids; △—△, phospholipids without protein.

Fig. 5. Effect of increased ionic strength on aggregate formation from the commercial phosphatidylcholine liposomes with ovalbumin. To a mixture of ovalbumin (1.0 mg) and the liposomes (1.0 mg), a buffer giving the final ionic strength indicated was added and it was treated as described in Fig. 1.

factor for binding and aggregate formation of phospholipid liposomes with ovalbumin, the effect of ionic strength was studied by the use of the liposomes from the commercial phosphatidylcholine preparation. Buffers used were prepared as follows: To an equimolar mixture of Tris and sodium acetate giving the indicated final ionic strength, HCl solution of the corresponding concentration was added to adjust the pH to 2.5. An almost constant amount of phospholipid was found in the precipitate at a low ionic strength below $I = 0.15$. When the ionic strength of the buffer was increased to $I = 0.2$, there was a sharp drop in the amount of phospholipids aggregated (Fig. 5). This fact indicates that a charge–charge interaction was essential for the binding and aggregation between the phospholipids and ovalbumin. The binding of cytochrome *c* to phosphatidylserine liposomes was inhibited by increase in the ionic strength from 0.01 to 0.15 M KCl[11], while the binding of bovine serum albumin to the mixed phospholipid liposomes consisting of phosphatidylcholine, cholesterol and dicetyl phosphate was slightly decreased in 0.2 M NaCl[6]. The present result shows a close similarity with the former finding on the effect of ionic strength; the latter data on bovine serum albumin demonstrated the presence of salt-stable association of the protein with the liposomes. However, this latter phenomenon was not observed in our studies.

Complex formation of cytochrome *c* with phospholipids was prevented by bivalent cations[17], while triphosphoinositide formed a complex with serum albumin in the presence of Ca^{2+} (ref. 18). Thus, it is of interest to see what effect a bivalent cation, Ca^{2+}, has on binding and aggregation of ovalbumin to the liposomes from the commercial phosphatidylcholine. As can be seen in Fig. 6, when the Ca^{2+} concentration increased to 6 mM, precipitation of phospholipids was almost completely prevented. This finding indicates that ovalbumin with a positive charge at pH 2.5 does not bind with liposomes by calcium aslt formation of phosphate groups in their particles.

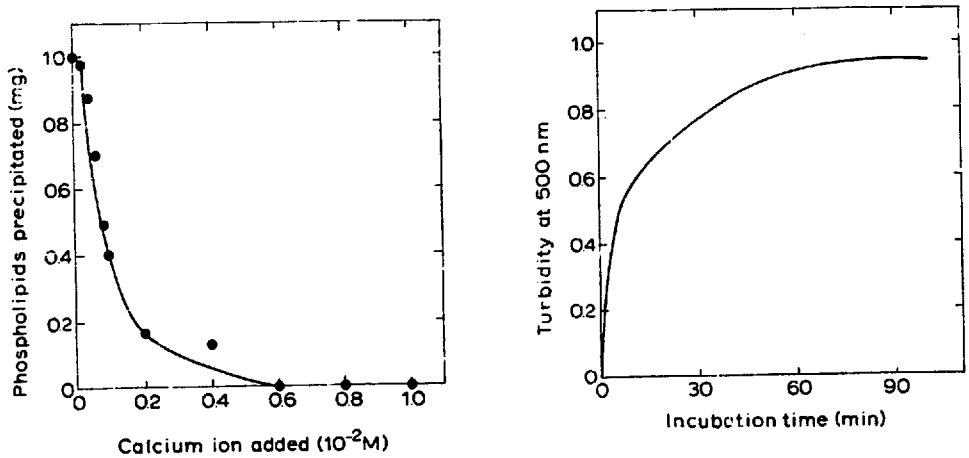

Fig. 6. Effect of Ca^{2+} concentration on the aggregate formation. To a mixture of ovalbumin (1.0 mg) and the commercial phosphatidylcholine liposomes (1.0 mg), the buffer containing $CaCl_2$ of the final concentration indicated was added and it was treated as described in Fig. 1.

Fig. 7. Time dependence of the aggregate formation. To a cell, 0.1 ml of 2% phosphatidylcholine liposomes, 3.88 ml of the second buffer (pH 2.5), and 0.12 ml of 1% ovalbumin were added. Turbidity increase was recorded at 500 nm referred to another cell without protein.

The time-course curve of aggregation of liposomes from commercial phosphatidylcholine with ovalbumin is presented in Fig. 7. For this examination, an increase in turbidity based on aggregate formation of the lipid particles with the protein molecule was followed by light scattering at 500 nm. The interaction between the protein and the liposomes proceeded very rapidly for the first 1 min and then gradually with time. After standing for 90 min, the increase in turbidity of the solution reached a maximum level. The turbidity decreased considerably after 2 h, due to settlement of the aggregate formed to the bottom of cuvette. The above fact shows that the aggregation of liposomes with ovalbumin depends on time.

The aggregate formed was further treated with buffers to obtain some information on the mechanism of interaction between ovalbumin and the liposomes, and the results are shown in Table III. A small amount of phospholipids was extracted in each supernatant obtained by centrifugation after incubation at 37 °C for 1 h at pH 2.5. A considerable amount of phospholipids from the aggregate was recovered in the supernatant at pH 6.0 with stepwise decrease in the number of extractions.

TABLE III

PHOSPHOLIPIDS EXTRACTION FROM THE AGGREGATE WITH THE BUFFER

An aggregate was prepared from a mixture of 2 mg of ovalbumin and 2 mg of the commercial phosphatidylcholine liposomes. The aggregate, resuspended in 4.0 ml of the first buffer, was centrifuged after incubation at 37 °C for 1 h.

Extraction No.	*Turbidity at 280 nm*		
	pH 2.5	*pH 2.5**	*pH 6.0*
1st	0.057	0.079	0.667
2nd	0.062	0.255	0.512
3rd	0.066	1.155	0.352
4th	0.071	0.139	0.160
5th	0.077	0	0

* Sonicated for 1 min before incubation.

After 5 extractions at pH 6.0, visible precipitate was not found on the bottom of the tube. Quantitative analysis of phospholipids in the tube gave a negative result, indicating that all of the phospholipids in the initial aggregate was recovered in the supernatant after 5 extractions at pH 6.0. The ovalbumin molecule with a positive charge at pH 2.5 would be converted to a negative one at pH 6.0, at which the liposomes were also negatively charged due to the presence of phosphatidic acid and phosphatidylethanolamine as contaminants in the preparation. Because of electrostatic repulsion between the two components, the liposomes released may be dispersed into the buffer (pH 6.0). When the aggregate was submitted to sonic oscillation at pH 2.5 and centrifuged after the incubation, a maximum amount of the phospholipids was found in the supernatant obtained from the third extraction. After 5 treatments, no phospholipids remained in the tube, as in the extraction at pH 6.0. Although it is not clear at present why phospholipids become extractable by sonication at pH 2.5, it is plausible that the aggregation is inhibited by the change from inter- to intra-micellar binding of the ovalbumin molecule, followed by reconstruction of the initial liposomes on sonication.

DISCUSSION

Liposomes formed ultrasonically from the commercial phosphatidylcholine preparation were stable and resistant to storage for 1 month in a refrigerator without measurable change in turbidity. They did not form any precipitate with incubation and low-speed centrifugation after dilution with buffers (pH 1.5–10.0). The liposomes from pure phosphatidylcholine precipitated by similar treatment at pH 2.5 to the extent of approximately 40% of the amount added. A part of the phosphatidylethanolamine was also recovered in the precipitate obtained from its liposomes, while phosphatidic acid dispersions did not form any aggregate after treatment. Sounders *et al.* reported that isotropic dispersions (15%, w/w) prepared from phosphatidylcholine gave a stable clear solution by sonic oscillation[19]. In our experiments, the liposomes from 2% phospholipid dispersions by sonic irradiation were diluted 20-fold with the buffer (pH 2.5) before the incubation. Phospholipid liposomes from phosphatidylethanolamine or phosphatidylcholine may, thus, have a tendency to aggregate at such a low concentration because of the relatively low viscosity of the medium. The liposomes consisting of a mixture of phosphatidic acid and phosphatidylcholine formed little precipitate above 10% phosphatidic acid in phosphatidylcholine after such a treatment. The liposomes of phosphatidylethanolamine and phosphatidylcholine precipitated to a small extent, in spite of the recovery of a considerable amount of phospholipids in the aggregate from its individual liposomes. The liposomes containing 5% of both phosphatidic acid and phosphatidylethanolamine in phosphatidylcholine gave a small amount of precipitate which was lower than the average amount of 5% phosphatidic acid in phosphatidylcholine and 5% phosphatidylethanolamine in phosphatidylcholine. This fact indicates that either phosphatidic acid or phosphatidylethanolamine has an additional effect on suppression of the aggregation from phosphatidylcholine liposomes. The above fact also suggests that the aggregate formation from mixed phospholipid liposomes formed from these two or three components is lower than that from the individual phospholipid liposomes, except for phosphatidic acid. The commercial phosphatidylcholine preparation contained phosphatidic acid and phosphatidylethanolamine as major contaminants, and sphingomyelin and lysophosphatidylcholine as minor ones, as judged from the results of thin-layer chromatography. Phosphatidic acid and phosphatidylethanolamine contents of the preparation were found to be approx. 7.3 and 4.8%, respectively. Thus stability of the liposomes from the commercial phosphatidylcholine may be mainly attributable to the inhibition of aggregation by both phosphatidic acid and phosphatidylethanolamine present. Lyso-phosphatidylcholine present in phosphatidylcholine dispersions prevented the increase in turbidity after dilution with electrolyte solution[20]. The liposomes from a mixture of phosphatidylcholine and cholesterol were electromicroscopically dispersed to microparticles by the addition of lyso-phosphatidylcholine[21]. Phosphatidylcholine dispersions with a particle weight of $3.5 \cdot 10^6$ were disrupted into small units having an average particle weight of $5.4 \cdot 10^4$ by the addition of 0.1 M 1-anilinonaphthalene-8-sulfonate[22]. The above facts show that these agents act on disruption of phosphatidylcholine liposomes from large unit particles to small ones. For this reason, phosphatidic acid, a surface active agent, as well as lyso-phosphatidylcholine, has a protective effect on the precipitation of phosphatidylcholine liposomes, it may be

possible that liposomes with a large particle weight are converted into those having small units by the addition of phosphatidic acid. However, it is implausible that the liposomes from a mixture of phosphatidylethanolamine and phosphatidylcholine have small particle size, since the particle weight of phosphatidylethanolamine liposomes[23] was nearly the same as that of phosphatidylcholine liposomes[22]. Furthermore, our preliminary experiment showed that the liposomes from the commercial phosphatidylcholine had an apparent sedimentation coefficient of $s^\circ_{20,w} = 15$ S at 1.0% (pH 4.0, $I = 0.1$). This observation suggests that phosphatidylcholine liposomes containing phosphatidic acid and phosphatidylethanolamine also exist as large particles without lowering the unit weight of the liposomes from phosphatidylcholine alone. Further clarification of the situation must await detailed studies.

Ovalbumin made the liposomes from the commercial phosphatidylcholine precipitate at acidic pH values, but not at neutral or alkaline pH values, though the liposomes alone did not form any aggregate under such conditions. Since the commercial phosphatidylcholine contained some phospholipids as contaminants, it should be necessary to clarify what component in the phospholipids was responsible for its interaction with ovalbumin. None of the protein was detected in the precipitate obtained from each liposomes from pure phosphatidylcholine, phosphatidylethanolamine, or both. These results indicate that the protein binds neither with phosphatidylcholine nor phosphatidylethanolamine even under such acidic conditions. It is well known that basic proteins, such as cytochrome *c*, interact with dispersions from acidic phospholipids with their charge neutralization under nearly physiological conditions. Phosphatidylcholine liposomes with little negative charge at pH 7.6 (ref. 21), however, showed no interaction with cytochrome *c*[24]. Since ovalbumin has its isoelectric point at pH 4.6–4.8 (ref. 25), the protein molecule is positively charged at pH 2.5. At this pH, the net charge of phosphatidylcholine liposomes is slightly positive[21].

Thus, the binding of ovalbumin to phosphatidylcholine liposomes cannot be due to their charge–charge repulsion. Although phosphatidylethanolamine, an acidic phospholipid, is less positive than phosphatidylcholine in acidic solution, the difference between the net charges of phosphatidylethanolamine and ovalbumin may not be enough for their binding by electrostatic interaction because of the absence of binding of the protein to phosphatidylethanolamine liposomes.

In contrast to these liposomes from phosphatidylcholine and phosphatidylethanolamine, ovalbumin bound with all dispersions from phosphatidic acid alone and mixed phospholipid liposomes containing phosphatidic acid at pH 2.5. Since phosphatidic acid possesses a phosphate residue but no cationic group in its molecule, its dispersions always have a negative charge at pH values at which the primary and secondary phosphate groups dissociate. Because of a strong acidity of the primary phosphate group, positively charged ovalbumin may bind with phosphatidic acid dispersions with charge–charge interaction at pH 2.5. It should be noted that zwitterions composed of polyelectrolyte, such as proteins, precipitated with aggregation at the isoelectric point. Thus, aggregation may be facilitated with charge neutralization between phosphatidic acid dispersions and the protein. The aggregation was not observed in the presence of an excess of either phosphatidic acid or ovalbumin. This fact infers that aggregate formation is prevented by insufficient charge neutralization between phosphatadic acid and ovalbumin. It was pointed out that ribo-

nuclease, lysozyme and poly(L-lysine) caused a visible aggregate by binding to phosphatidylserine liposomes, but cytochrome *c* did not[11]. Thus, other factor(s) besides charge neutralization should be taken into consideration for these aggregation phenomena.

It was amply shown that liposomes formed from mixtures of phospholipids interacted with cytochrome *c*[6,11,17,24,26] and bovine serum albumin[16]. Our results presented here indicate that only phosphatidic acid is the component responsible for initial binding of ovalbumin in the mixed phospholipid liposomes. Thus, a principle responsible for interaction with ovalbumin may be phosphatidic acid in the liposomes from the commercial phosphatidylcholine, present in the preparation as a contaminant, and is not phosphatidylcholine or phosphatidylethanolamine. Bovine serum albumin bound only with the mixed phospholipid liposomes containing dicetyl phosphate[16]. Insulin was also strongly solubilized into the chloroform phase by phosphatidic acid and dicetyl phosphate[27]. These observations support the contribution of phosphatidic acid for binding of ovalbumin to the liposomes from the commercial phosphatidylcholine.

An amount of phospholipids above that of the phosphatidic acid added was found in the aggregate from the mixed phospholipid liposomes by the interaction with ovalbumin. Moreover, phospholipids were found in the supernatant from the aggregate formed after extraction with the buffer (pH 6.0). Our preliminary experiments also showed that a considerable amount of phospholipids was released in the supernatant by low-speed centrifugation after pepsin digestion of the aggregate at pH 2.5. These facts infer that drastic disorder of the liposomes with unilamellar structure does not occur in the process of aggregate formation due to binding of the protein.

Our other data indicated that pepsin digests of ovalbumin markedly lost the ability to form an aggregate from the commercial phosphatidylcholine liposomes (unpublished observation). This finding suggests that the size of protein molecule is an important factor for binding to the phospholipid liposomes. A study is currently in progress in our laboratory to provide further information on the nature of the interaction of protein and phospholipid liposomes.

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