Biochimica et Biophysica Acta, 982 (1989) 187–195
Elsevier

BBAMEM 74473

Comparison of phosphatidylethanolamine and phosphatidylcholine vesicles produced by treating cholate-phospholipid micelles with cholestyramine

S.P. Shi, Catherine C.Y. Chang, Gwyn W. Gould * and T.Y. Chang

Department of Biochemistry, Dartmouth Medical School, Hanover, NH (U.S.A.)

(Received 23 January 1989)

Key words: Phospholipid vesicle; Phosphatidylcholine; Phosphatidylethanolamine; pH dependence; Vesicle permeability; Cholate–phospholipid micelle; Cholestyramine

We have previously reported the preparation and characterization of unilamellar phosphatidylcholine vesicles from cholate-phospholipid micelles treated with the bile-salt sequestrant cholestyramine (Ventimiglia, J.B., Levesque, M.C., and Chang, T.Y. (1986) Anal. Biochem. 157, 323–330). We now describe a slightly modified procedure for forming unilamellar vesicles consisting of phosphatidylethanolamine, and the characterization of the resultant vesicles by gel exclusion chromatography. In contrast to phosphatidylcholine vesicles, the formation of phosphatidylethanolamine vesicles is highly pH dependent; pH 9.2 is superior to pH 8.1 or pH 7.1. Via the dialysis step, the final pH of the vesicles could be altered to be at 8.1 or at 7.1, although decreasing the pH from 9.2 resulted in the loss of approx. 20% of the total lipid as large aggregates. Residual cholate was still present in the resultant vesicles after cholestyramine treatment; the low levels of cholate, removable by dialysis, was found to stabilize the phosphatidylethanolamine vesicles formed at pH 8.1. These results suggest that the majority of the amino groups of the phosphatidylethanolamine molecules should either be in the deprotonated form, or be neutralized and/or restricted by the anionic cholate monomers in order to facilitate the vesicle formation. Phosphatidylethanolamine vesicles were found to be much more permeable to small ions than phosphatidylcholine vesicles. The incorporation of phosphatidylserine, but not phosphatidylinositol, into the phosphatidylethanolamine vesicles at 10% resulted in decreased permeability of the bilayer against the cobalt ion influx, suggesting cooperative and complementary packing of phosphatidylethanolamine and phosphatidylserine molecules within the bilayer.

Introduction

Vesicles are important tools to study the physical and chemical properties of membranes, in drug delivery systems, and for the study of lipid–protein interactions. A number of techniques for the preparation of unilamellar vesicles are available (for reviews, see Refs. 1–3). For most of the described procedures, phosphatidylcholine (PC) from various sources has been the major lipid species used. Under certain conditions, other classes of phospholipid such as phosphatidic acid [4], or the complex formed between phosphatidylserine (PS) and Ca^{2+} [5], or mixture of phosphatidylethanolamine (PE) and PS [6] have been shown to form unilamellar vesicles. In addition, Ho et al. [7] reported a novel method of preparing immunoliposomes consisting of PE and palmitoyl-immunoglobulin G.

PE plays an important role in determining the structure and/or functions of various biological membrane systems (for selected reviews, see Refs. 8 and 9). The unique, inverted cone-shaped configuration of the PE molecule renders the formation of unilamellar vesicles consisting of PE as the major lipid an unfavorable process. In fact, only one published procedure has clearly documented the successful formation of unilamellar vesicles consisting exclusively of PE. This method required prolonged and high-powered sonication of aqueous PE dispersions buffered at pH > 9, followed by ultracentrifugation to remove non-unilamellar material from the mixture.

* Present address: The Institute of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

Correspondence: T.Y. Chang, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756, U.S.A.

This laboratory previously reported the preparation and characterization of unilamellar vesicles composed of PC, from a cholate-phospholipid micellar solution treated with cholestyramine [11]. In this report, we extend this study and describe a slightly modified procedure to form unilamellar PE vesicles which is both rapid and reproducible. The size characteristics of the resultant PE vesicles were examined, and the vesicle preparation shown to be small (22 nm diameter at pH 9.1) and homogeneous. By introducing a dialysis step, it was possible to lower the pH of the vesicle suspension to pH 8.1 without sacrificing recovery. In addition, the ion permeability of these vesicles was examined using a fluorescence procedure to follow the time course of Co^{2+} quenching of a dye (calcein) trapped intravesicularly [13,14]. The results show that this procedure produces vesicles with ion permeabilities expected for pure PE. This procedure should be useful for the study of PE-lipid and/or PE-protein interactions. As a first step, we report that the inclusion of low amounts of PS (but not PI) greatly decrease the permeabilities of these vesicles to divalent cations, suggestive of a cooperative interaction of these species in the membrane.

Material and Methods

Materials

Cholestyramine was from Bristol-Myers Company, Evansville, IN. It was washed four to five times with H_2O, dried via lyophilization to powder form, and stored at room temperature. Sodium cholate was from Cal-Biochem. [^{3}H]Cholic acid was from New England Nuclear. Sephacryl S-1000 was from Pharmacia. Alumina (type WN-6, neutral), cholesterol, ethanolamine-hydrochloride, glycerol, calcein, and other chemicals were from Sigma. Silica gel used for column chromatography (Davisil 62) was purchased from Davison Chemical (Baltimore, MD). Thin-layer chromatography (TLC) plate (Silica gel-H) was purchased from Fisher (catalog No.: 84101). All organic solvents were spectro-analyzed grade from Fisher.

Methods

Purification of PC, PE, and analyses of various phospholipids. Crude phosphatidylcholine (PC) was prepared from egg yolk by the modified method of Singleton et al. [15]. Crude PC (containing ≈ 20% PE and other minor phospholipids) was purified to homogeneous PC through two successive column chromatographic steps: For 4 g of crude PC, approx. 100 g of silica gel (activated by heating at 130°C for 12 h followed by cooling at room temperature), mixed with $CHCl_3$ as thick slurry, was packed into a glass column (2.8 cm diameter). The column was washed with five column volumes of $CHCl_3$. Crude PC dissolved in $CHCl_3$ was applied and eluted with 300 ml per fraction of stepwise gradient mixtures consisting of $CHCl_3$ and increasing concentrations of CH_3OH. PE fractions eluted at approx. 10–15% CH_3OH in $CHCl_3$, followed by PC which began to be eluted at approx. 30% CH_3OH in $CHCl_3$. The PC-containing fractions were concentrated to near dryness via rotary evaporation at ≈ 10°C, redissolved as a concentrated solution in $CHCl_3$. This fraction was devoid of PE, but contained sphingomyelin and other minor phospholipids such as PS as impurities. The PC-containing fraction was further purified using neutral alumina according to Singleton et al. [15]. The procedure described above was performed in darkness. The resultant PC proved to be at least 98% pure by various TLC criteria, and was colorless. Stored as approx. 100 mg/ml solution in 90% $CHCl_3$/10% CH_3OH at −20°C in the dark under argon, it remained to be pure and colorless for at least 3 months.

PE used for all experiments reported in this manuscript was prepared by transphosphatidylation via phospholipase D from homogeneous egg PC according to a published procedure [16]. The PE thus obtained was dissolved in $CHCl_3$ and was purified using silica gel column chromatography performed at room temperature in the dark (3 g PE vs. 90 g silical gel). PE thus obtained was at least 98% pure by various TLC criteria, and was colorless. Stored in the dark at −20°C under argon, it remained pure for four or five days; beyond this time, lysophosphatidylethanolamine became detectable, amounting to approx. 5% of total phospholipid in two or three weeks. For all the experiments reported here, PE was used within one week after purification by column chromatography.

Egg yolk phosphatidylglycerol (PG) and bovine phosphatidylinositol (PI) were from Sigma, while bovine phosphatidylserine (PS) was from Avanti Polar-Lipids, Inc. TLC analyses showed at least 98% purity for each of these three lipids.

Preparation of detergent-lipid solutions. The cholate-lipid solutions were prepared by slight modifications of the method described in Ventimiglia et al. [11]: Phospholipid was added to a thin, glass tube (13 × 100 mm borosilicate). After evaporation of organic solvent under nitrogen and lyophilization in the dark overnight, enough cholate and 50 mM Tri-HCl, 10 mM EDTA (pH 7.2–9.2), or 10 mM borate, 1 mM EDTA, pH 9.2, was added to give a final volume of 2 ml containing 10 mg/ml phospholipid and 20 mg/ml cholate (49 mM). The mixture was purged with argon, capped with a rubber plug, sealed with parafilm layers, and sonicated in the dark at 4°C. A bath-type Branson ultrasonic cleaner (Model B-12) was used for sonication; approx. 30 min was needed to reach transparent clarity for the mixture containing PE. After sonication, TLC analyses showed that up to 7% of PE was decomposed to lyso-PE and free fatty acid. The sonication time required for

clearing the solution containing PC was always less than 30 min; TLC analyses showed that less than 2% of PC was decomposed to lyso-PC.

Vesicle formation by cholestyramine treatment. The procedure was the same as that described previously [11] with one major modification: immediately before use, the dry cholestyramine power was washed twice with the same buffer used in the preparation of the cholate-lipid solution (2 ml buffer per 100 mg cholestyramine); after rapid washing by brief vortexing, the buffer was removed by centrifuging the mixture at $2000 \times g$ for 15 min in Beckman TJ-6 centrifuge for 10 min and discarding the supernatant. The pelleted cholestyramine slurry was mixed with cholate-lipid solution as described previously [11].

Sephacryl S-1000 column chromatography. The columns were packed, washed, and performed as described previously [11].

Separation of phospholipids by TLC. PC, PE, PG, PS, and PI were separated by TLC using the solvent system $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25 : 15 : 4 : 2, v/v). PE, PC, and PG were separated by TLC using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (170 : 25 : 25 : 6, v/v). PE, PS, PI and lyso-PE were separated by TLC using $CHCl_3/CH_3OH/28\%\ NH_3$ in H_2O (65 : 35 : 5, v/v). After TLC, the resolved bands were visualized by lightly exposing the plate to iodine vapors; the appropriate bands were scraped, extracted with $CHCl_3/CH_3OH$ (2 : 1, v/v), and assayed for phospholipid.

Permeability of PE and PC vesicles. This was determined by measuring the rate of fluorescence quenching of intravesicular calcein caused by cobalt ion entry into the vesicle [13,14]. A Hitachi MPF-2A fluorescence spectrophotometer was used for this purpose (excitation wavelength 490 nm; emission wavelength 520 nm). Vesicles were produced by cholestyramine treatment of cholate-lipid solution composed of 1.5 mg cholesterol. 40 mg cholate, and 20 mg PE or PC formed in 2 ml of 10 mM borate buffer (pH 9.2) containing 50 mM sucrose and 80 μM calcein. After the cholestyramine treatment, vesicles were dialyzed for 4 h in 100-fold volume of 10 mM borate buffer containing 50 mM sucrose (pH 9.2), to remove the external calcein, followed by centrifigation at $78000 \times g$ for 1 h at room temperature to collect the supernatant. Control experiments indicated that during the vesicle preparation step, more than 95% of the calcein included in the lipid-detergent solution was adsorbed onto the cholestyramine resin. In order to provide reliable fluorescent signals in the resultant vesicles, 80 μM calcein was the minimal concentration required for the purpose of detection (data not shown). The low concentration of Co^{2+} used (33 μM final) is considerably lower than the concentration of Ca^{2+} (3–5 mM) known to induce aggregation in PE/acidic lipid vesicles [17]. At this concentration, we do not expect Co^{2+} to induce any bilayer perturbing effects, since the effect of Ca^{2+} on pure PE vesicles has been shown to be limited [18].

Results

In experiments not shown, we find that the wet, buffer-washed cholestyramine pellets in slurry form (see Materials) is superior to the dry powder form employed in our previous work [11] in two aspects: (i) the recovery of vesicles after centrifugation was considerably greater and reproducible, and (ii) the average size of the resultant vesicle was smaller and the size distribution was more homogeneous. For these reasons, all experiments reported here involved the use of wet, buffer-washed cholestyramine pellets.

Fig. 1A shows that, at pH 8.5, increasing doses of cholestyramine were effective in absorbing cholate from the cholate-PE micelles such that after removing the resin by centrifugation, the cholate concentration in the supernatant (referred to as the first-treated supernatant) is reduced to approx. 10% of its initial concentration. This figure also shows that, using 50 mg of cholestyramine for 1 ml of cholate-lipid solution, at least 70% of the PE is recovered in the supernatant after the cholestyramine removal. Fig. 1B shows the increase in molar ratio of PE/cholate in the first-treated supernatant in response to increasing amounts of cholestyramine.

The first-treated supernatant was isolated and treated again with increasing amounts of cholestyramine. The mixture was centrifuged to isolate the supernatant (referred to as the second-treated supernatant) and analyzed for cholate, and for PE content. The results obtained (Fig. 2A, B) are qualitatively very similar to those shown in Figs. 1A, B; they are also similar to those previously reported for the PC-cholate micellar solution [11]. At 30 mg cholestyramine, the molar ratio of PE to cholate in the resultant supernatant is approx. 7. These experiments show that, for 1 ml of PE-cholate micelles (10 mg PE), the optimal dose of cholestyramine used for the first and second treatments are at 50 mg and 30 mg, respectively.

We next examined the effect of pH on recovery of PE and cholate in the supernatant after the cholestyramine treatment. Fig. 3A shows that, while the %PE recovered in the first-treated supernatant is independent of differences in pH, the recovery in the second-treated supernatant is very poor when the buffering pH falls below 8. This observation was reproducibly seen in three separate experiments. The control experiments (Fig. 3B, D) using the PC-cholate micelles show that the differences in pH do not significantly affect the recovery of PC vesicles.

After the second cholestyramine treatment, the supernatants recovered at pH 8.1 or at 9.2 were individually examined by gel exclusion chromatography on Sephacryl S-1000. Fig. 4A shows that, for PE-cholate solu-

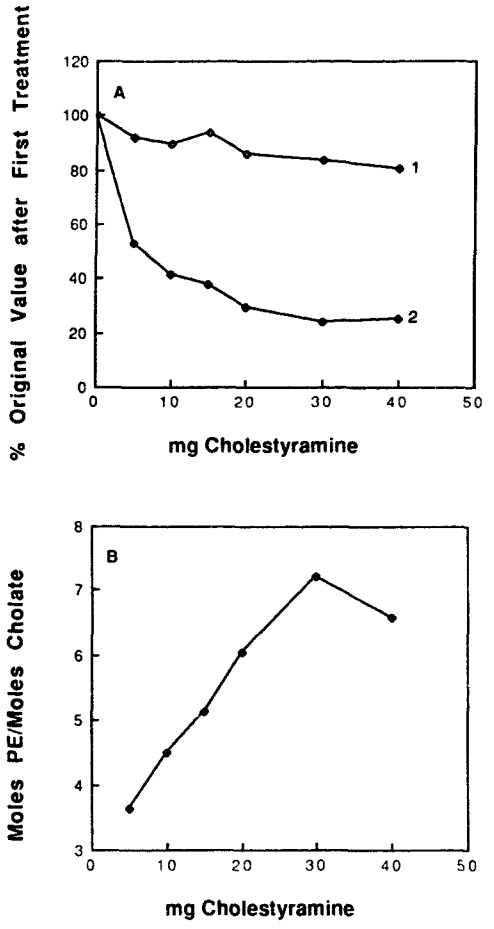

Fig. 1. The first treatment of cholate-PE solution with varying amounts of cholestyramine. One ml of ^{3}H-labelled cholate (49 mM) -PE (10 mg/ml) solution containing 50 mM Tris-HCl, 5 mM EDTA at pH 8.5, was treated with the indicated amounts of cholestyramine, mixed, then centrifuged. Aliquots were withdrawn for ^{3}H scintillation counting and for phospholipid analysis [31]. Symbols used: (A) 1, ◇, PE concentration; 2, ◆, cholate concentration. (B) ◆, molar ratio of PE to cholate in the first-treated supernatant.

tion at pH 9.2, essentially all of the material recovered is composed of small, unilamellar vesicles. The size of these resultant vesicles gradually increased when the buffering pH was decreased from 9.2 to 8.1 (Table I), with concurrent accumulation of material much larger in size ($\geq$ 2500 Å in diameter; Ref. 12) emerging at or near the void volume of the column. These results suggest that at pH below 8.1, non-vesicular aggregated material forms, the majority of which are pelleted along with the cholestyramine pellets during centrifugation, thus explaining the poor recovery in preparing the PE vesicles observed at pH 7.8 (Fig. 3A). The control experiments using PC-cholate (Fig. 4B, Table I) show that within the indicated range of pH values, the resultant material in the supernatant is essentially all unilamellar vesicles.

From the results shown in Figs. 2A, B, it is clear that residual cholate is still present in the supernatant after the second cholestyramine treatment. This reisdual cholate is not removed by a third cholestyramine treatment (data not shown). However, dialyzing the supernatants against excess amounts of buffer at room temperature for 4 h decreased the residual cholate content to essentially undetectable levels ($\leq$ 0.1% of its initial concentration). When PE vesicles prepared and dialyzed at pH 9 were briefly centrifuged once at 12000 × g for 20 min (to remove large aggregates), then analyzed by exclusion chromatography, it was found that they consisted almost entirely (approx. 98%) of small unilamellar

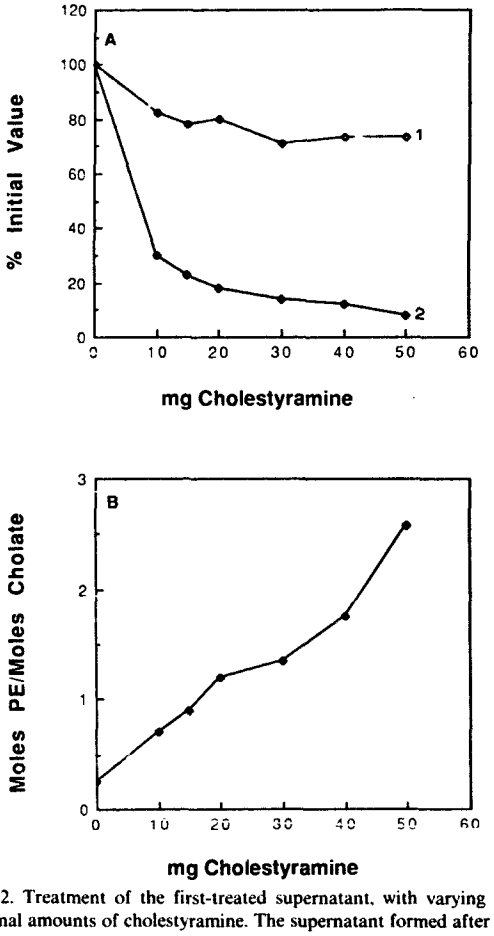

Fig. 2. Treatment of the first-treated supernatant, with varying additional amounts of cholestyramine. The supernatant formed after the first cholestyramine treatment (by treating 1 ml of cholate-PE solution with 50 mg of cholestyramine) had a pH value of 8.5 and a PE concentration of 8.5 mg/ml. Methods used for additional cholestyramine teatment, for analyses, and symbols used, were the same as described in Figs. 1A and 1B, except that in Fig. 2A, points are plotted as percentages of their original values in the first-treated supernatant.

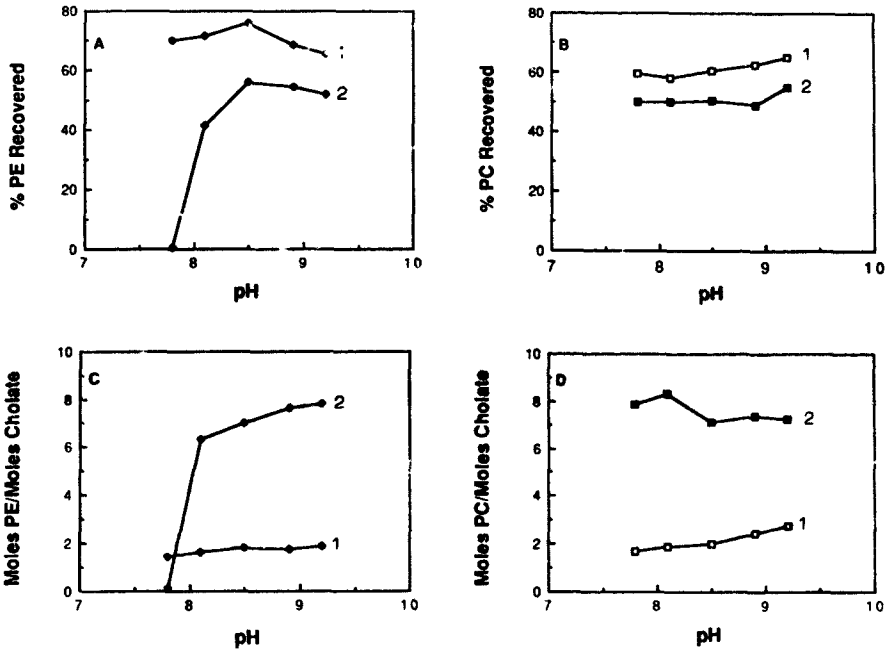

Fig. 3. The effect of pH on PE/PC recovery. The effect of pH values on PE recovery (A), on PC recovery (B), on PE/cholate molar ratio (C), and on PC/cholate molar ratio (D) present in the supernatant after the first cholestyramine treatment (presented as open symbols, curve 1), or after the second cholestyramine treatment (presented as closed symbols, curve 2), is presented. 1 ml of cholate (49 mM), PE or PC (10 mg/ml), cholesterol (0.75 mg/ml) mixed micellar solution was treated with 50 mg cholestyramine (for the first treatment), or with 30 mg cholestyramine (for the second treatment) as described under Methods. Buffers containing 50 mM Tris-HCl and 5 mM EDTA at various indicated pH values were used. The percent phospholipid recovered after the first or the second cholestyramine treatment was calculated based on using the starting material (10 mg) as 100%.

vesicles, which were stable at 4°C for at least two days (Fig. 5A). However, when this experiment was performed at pH 8.1, essentially all of the detectable lipid eluted at or near the void volume (Fig. 5B). The difference in size characteristics of vesicles shown in Fig. 4A and Fig. 5B can be explained by the difference in amount of residual cholate present in these vesicles (see Discussion). The poor recovery in lipid exhibited in Fig. 5B was due to the fact that, at pH 8.1, the dialysis step caused aggregation of lipid, most (approx. 75%) of which was pelleted and removed by centrifuging at $12000 \times g$ for 20 min.

We next prepared PE vesicles at pH 9.2, dialyzed them against buffer either at pH 7.1 or at pH 8.1, and analyzed the size distribution of the dialyzed material by gel exclusion chromatography. Figs. 6A, B show typical results obtained at pH 8.1. Approx. 78% of the material remained as small, unilamellar vesicles, while the rest of the material eluted at the void volume, presumably as a result of aggregation (Fig. 6A). Centrifugation at $80000 \times g$ for 1 h completely removed this aggregate as pellets; the material remaining in the supernatant after centrifugation consisted entirely of small, unilamellar vesicles (Fig. 6B). These vesicles were stable at 4°C for at least 2 days. Very similar results as those shown in Figs. 6A, B were obtained from experiments performed at pH 7.1. Control experiments using PC vesicles formed at pH 9.2 showed that the unilamellar

TABLE I

The average diameters of unilamellar PE and PC vesicles at different pH values

Nature of phospholipid	pH	$V_{e,avg}$ [a] (ml)	Diameter [b] (nm)	% recovered as unilamellar vesicles [c]
A. PE	8.1	40.5	42	82
	8.5	42.5	30	89
	9.2	44.3	22	100
B. PC	8.1	44.3	22	100
	8.5	44.6	20	100
	9.2	42.5	30	100

[a] $V_{e,avg}$ is the average elution volume for each vesicle type calculated on the weight-average basis from the elution profiles shown in Figs. 4A, B, according to the equation: $V_{e,avg.} = \Sigma(V_{e,i} \cdot [\text{PE or PC}]_i)/\Sigma[\text{PE or PC}]_i$, where the summation takes place over all i subpopulations of discrete sized vesicles with discrete PE or PC concentrations [11].

[b] Calculated from V_e according to Reynolds et al. [30].

[c] These values are obtained from elution profiles shown in Figs. 4A, B according to the equation: % = area of PE or PC peak behind the column void volume/total area of PE or PC peaks recovered from the column.

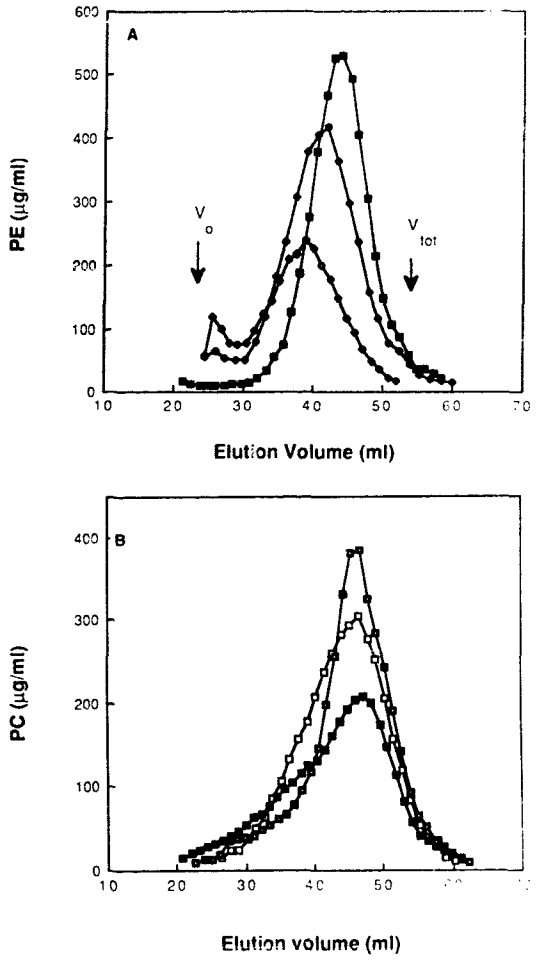

Fig. 4. Elution profiles of PE (A) and PC (B) vesicle on the Sephacryl S-1000 column at various pH values. PE and PC vesicles were prepared in 50 mM Tris-HCl buffer and 5 mM EDTA at different pH values by the treatment of cholestyramine. The buffers used for elution were the same as those used in preparing the vesicles. For each collected fraction, 100 μl aliquots were withdrawn for phosphate assay as described under Methods. For each experiment, the recovery of total phospholipid off the column varied between 90 and 99%. For this and other figures which involve the use of Sephacryl S-1000 column chromatography, the recovery of phospholipid off the column was calculated based on using the amount of phospholipid loaded onto the column as 100%. Symbols used: (A) ⊞——⊞, pH 9.2; ◆——◆, pH 8.5; ◇——◇, pH 8.1. (B) ■——■, pH 9.2; ◘——◘, pH 8.5; □——□, pH 8.1.

nature of the resultant PC vesicles did not change upon dialyzing the vesicles at pH 7.1, 8.1, or at 9.2 (results not shown).

It was previously shown that the cholestyramine treatment could successfully produce unilamellar PC vesicles containing up to 10% of negatively charged phospholipids such as PI, PS, or PG [11]. We now find that at pH 9.2, this method can produce similar mixed vesicles using PE as the major phospholipid (Fig. 7). In addition, we find that PS is evenly distributed among various size populations of the mixed vesicles using

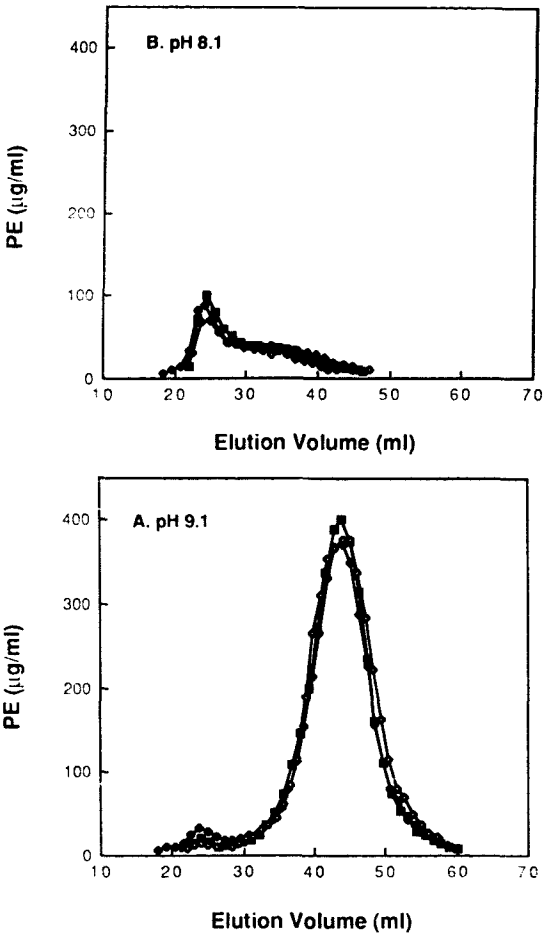

Fig. 5. The elution profiles of cholestyramine-produced PE vesicles formed and dialyzed at pH 9 (A) or at pH 8.1 (B), then stored for different indicated time at room temperature. PE vesicles were prepared as described under Methods in 50 mM Tris-HCl + 5 mM EDTA buffered at pH 9 or at pH 8.1, dialyzed at room temperature against 100-fold volume of the same buffer for 4 h, spun at 12000 × g for 20 min in a Sorvall centrifuge using Sorvall rotor SS34 to remove large aggregates. For samples prepared and dialyzed at pH 8.1, approx. 75% of total phospholipid was pelleted by the low-speed centrifugation step, while for samples prepared and dialyzed at pH 9, less than 5% of total phospholipid was pelleted under the same condition. The supernatants recovered after the centrifugation were stored at room temperature for different time before analyses by the Sephacryl S-1000 column chromatography. Using the amount of phospholipid loaded onto the column as 100%, the recovery of phospholipids averaged 98% for experiments shown in A, and averaged 67% for experiments shown in B. Symbols used: ◇——◇, no storage time; □——□, 24 h; ◆——◆, 48 h.

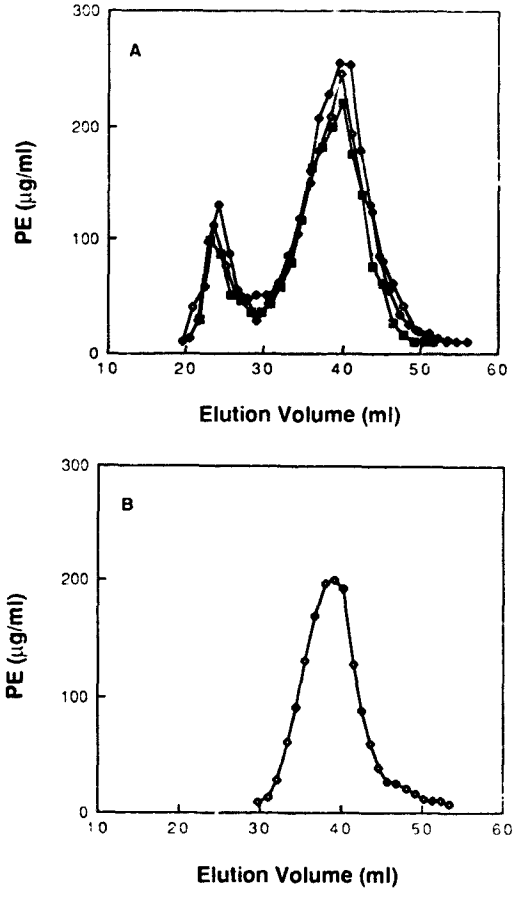

Fig. 6. (A) The elution profile and stability of PE vesicles formed at pH 9.2 and dialyzed at pH 8.2. The PE vesicles were prepared in 50 mM Tris-HCl + 5 mM EDTA (pH 9.2) by the treatment of cholestyramine, then dialyzed at room temperature for 4 h against 100-fold volume of 50 mM Tris-HCl + 5 mM EDTA buffered at pH 8.2, spun at $12000 \times g$ for 20 min. The supernatant obtained was stored at room temperature for time indicated prior to analyses by the Sephacryl S-1000 column chromatography. The recovery of total phospholipid averaged approx. 90% for the three experiments shown. Symbols used were the same as described in Fig. 5. (B) Removal of non-unilamellar material by high-speed centrifugation. The PE vesicles were prepared as described in (A), except that after dialysis, the vesicles were spun at $78000 \times g$ for 1 h at room temperature, the recovered supernatants were analyzed by Sephacryl S-1000 column chromatography without storage. The recovery of total phospholipids was approx. 88%.

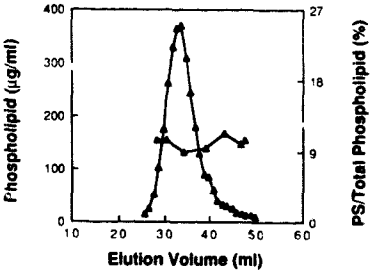

Fig. 7. The elution profile (Δ———Δ) and lipid composition (▲———▲) of mixed vesicles produced by the cholestyramine treatment. 2 ml of mixed micellar solution containing 40 mg cholate, 10 mg PE, 1.8 mg PS, and 1.5 mg cholesterol in 50 mM Tris-HCl, 1 mM EDTA (pH 9.2), was treated with cholestyramine, and centrifuged at $78000 \times g$ for 1 h at room temperature. The supernatant (1.0 ml, 4.8 mg phospholipid/ml) was analyzed by the Sephacryl S-1000 column chromatography. Aliquots from each fraction were analyzed for total phospholipid content as well as for PE and PS content as described under Methods. The recovery of total phospholipids off the column was approx. 88%

either PE or PC as the major phospholipid. Similar results were recorded using PI or PG (data not shown). The result using PE as the major phospholipid, representative of all of the experiments described above, is shown in Fig. 7. We next investigated and compared the permeability characteristics of the PE and the PC vesicles produced by cholestyramine using a fluorescence procedure [13,14]. The time course of cobalt ion entry was monitored by its ability to quench the fluorescent dye calcein trapped intravesicularly. A typical result is shown in Figs. 8A, B. Control experiments showed that Triton X-100 caused immediate rupture of the vesicles and the rapid and complete quenching of the fluorescence, indicating the intravesicular nature of the dye prior to Triton X-100 treatment.

Addition of Triton X-100 in the absence of $CoCl_2$ had no effect on the fluorescence intensity (data not shown). The half-time for Co^{2+} entry into PE and PC vesicles is presented in Table II. These values agree closely with data from other laboratories [14], and show that in general, PE vesicles are more permeable than PC vesicles. Interestingly, the inclusion of PS at 10 mol% resulted in a significant decrease in the permeability of the PE vesicles (206 min compared to 15 min). Control experiments showed that PS had little or no effect on the permeability characteristics of the PC vesicles (data not shown).

Discussion

This paper describes a rapid, reproducible and efficient method for preparing PE vesicles. The time required for sonication and for cholestyramine treatment

TABLE II

The permeability of PE and PC vesicles to cobalt II ions

Lipid composition	Half-time of cobalt ion entry rate (min)
100% PC	75 ± 7 ($n = 4$)
100% PE	15 ± 3 ($n = 6$)
90% PE + 10% PS	202 ± 76 ($n = 3$)
90% PE + 10% PI	16 ± 3 ($n = 3$)

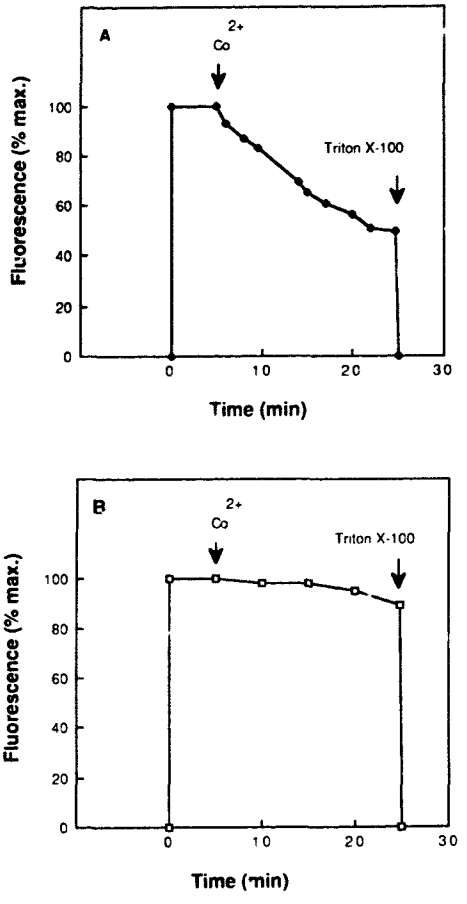

Fig. 8. Permeability of PE (A) and PC (B) vesicles to cobalt II ions. At zero time, 200 μl of the aqueous vesicle suspensions prepared as described in Methods, were added to a cuvette which contained 2.8 ml of the buffer (10 mM borate buffer with 50 mM sucrose at pH 9.2). After 5 min of incubation at room temperature, 40 μl of 2.5 mM of $CoCl_2$ in H_2O was added (final concentration: 33 μM). Fluorescence intensity was measured at various time intervals. Finally, 168 μl of 10% Triton X-100 was added to the cuvette at the 25th min to destory the integrity of the vesicles. Separate control experiments showed that Triton X-100 added 1 min after the $CoCl_2$ addition also caused the immediate and complete quenching of calcein fluorescence, indicating that the only source of fluorescence was intravesicular calcein.

is less than 2 h. The dialysis time (4 h) (which is required to completely remove the residual cholate, and/or to alter the final pH of the preparation), is the only time-consuming step. Modified from an original method previously reported from this laboratory [11], this method also enables the preparation of unilamellar PC vesicles in higher recovery and with greater reproducibility.

The process of PE vesicle formation, unlike that of PC vesicle formation, was found to be highly pH dependent: pH 9.2 resulted in higher lipid recoveries and a more homogeneous size distribution than either pH 8.1 or pH 7.1. Vesicles prepared at pH 9.2 are stable at 4°C for at least 2 days. Via the dialysis step, the final pH of the vesicles could be canged, although decreasing the pH from 9.2 resulted in approx. 20% of the total material being excluded from Sephacryl S-1000, presumably this material represents aggregates of PE vesicles [19]. This aggregate was easily removed from the vesicle preparation by ultracentrifugation (Fig. 6B); the exact nature of this material was not further investigated. The above observations concerning the pH dependence of PE vesicle formation is consistent with the earlier observation made by Stollery and Vail [10] using a completely different method to form the PE vesicles, and can be explained by the tendency of PE to adopt the hexagonal phase at neutral pH, due to poor head group hydration [9,19,20]. Taken together, these results strongly suggest that the majority of the amino groups of the individual PE molecules need to be in the deprotonated form in order to facilitate the PE vesicle formation, since the apparent pK_a of the $-NH_3^+$ group for pure PE vesicles is known to be at approximately pH 7.2 [19]. Once the vesicle is formed, the deprotonated form of the PE molecule does not seem to be a stringent requirement for maintaining the vesicle configuration. It is also worthwhile to note that low levels of residual cholate, not removable from the vesicles by extensive cholestyramine treatment, greatly stabilize the PE vesicle configuration at pH 8.1, since removing the residual cholate by dialysis led to complete destruction of the unilamellar vesicles (Figs. 4, 5). This observation is consistent with that of Madden and Cullis who also report a stabilization of PE bilayers by low levels of detergent [21]. The stability of PE bilayers has been shown to be modulated by water-soluble components which affect the solvation of the headgroup (e.g. NaCl, Ref. 22; chaotropic reagents, Ref. 23; sugar alcohols, Ref. 24; and other anions/cations, Ref. 25). We therefore suggest that this effect of cholate may be as a result of interacting and changing the hydration of the PE headgroup, thus neutralizing and/or restricting the bilayer-disturbing property of the latter moieties. Parallel experiments showed that removal of the residual cholate by dialysis did not destory PC vesicles formed at pH 8.1 or at 8.5 (results not shown).

We find PE vesicles to be much more accessible towards small ion influx than the PC vesicles formed under the same condition (Fig. 8; Table II). This is not surprising since the PE monomer, known to have an inverted cone-shaped configuration, is expected to be not as compatible in forming tight, intermolecular packings within the bilayer as PC [9,22]. It is interesting to note that the incorporation of PS into the PE vesicle at approx. 10 mol% resulted in a much reduced permeability of the bilayer against cobalt ion influx (Table II). Previously, using the technique of ^{31}P-NMR, Tilcock and Cullis [26] showed that the inclusion of PS at 15

mol% or more of total phospholipid stabilized the bilayer organization of aqueous dispersions of PE-PS systems. The effect of PS was tentatively attributed to its anionic (acidic) property [27]. Similar observations were also reported by Wang and Huang [28] but at higher PS/PE ratios (1.1). However, in our system, the data in Table II clearly demonstrate that the vesicle stabilizing effect of PS is not simply the result of negative charge, as PI did not change the permeability of the PE vesicles to Co^{2+}. It has been suggested that the polar group of PS in the fully charged state at neutral pH allows tighter packing in the bilayer than the bulkier glycerophosphocholine group of PC [29]. By analogy, that the stabilizing effects of PS, but not PI, on PE vesicles observed in our system may be governed by the size of the headgroup bearing the negative charge. The general applicability of this interpretation needs to be tested by further experimentation.

In conclusion, the preparation and characterization of PE vesicles by a novel procedure reported here has provided some interesting insights regarding the physico-chemical form of PE molecules in the bilayer. The results described in this manuscript may facilitate the study of PE biochemistry by allowing rapid and reproducible formation of PE vesicles.

Acknowledgements

We were informed of the advantages of using wet cholestyramine pellets in slurry form for vesicle production by Dr. E. Dawidowicz of Tufts University Medical School, through personal communication. We thank Mary Harrington for careful typing of the manuscript. This work was supported in part by NIH grant HL 36709.

References

1 Gregoriadis, E. (ed.) (1984) in Liposome Technology, Vol. 1, CRC Press, Boca Raton, FL.
2 Szoka, F. and Papahadjopoulos, D. (1980) Ann. Rev. Biophys. Bioeng. 9, 467–508.
3 Hauser, H. (1982) Trends Pharmacol. Sci. 3, 274–277.
4 Hauser, H. and Gains, N. (1982) Proc. Natl. Acad. Sci. USA 79, 1683–1687.
5 Papahadjopoulos, D., Vail, W.J., Jacobson, K. and Poste, G. (1975) Biochim. Biophys. Acta 394, 483–491.
6 Szoka, F., Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
7 Ho, R.J.Y., Rouse, B.T. and Huang, L. (1986) Biochemistry 25, 5500–5506.
8 De Kruijff, B. (1987) Nature 329, 587–588.
9 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.
10 Stollery, J.G. and Vail, W.J. (1977) Biochim. Biophys. acta 471, 372–390.
11 Ventimiglia, J.B., Levesque, M.C. and Chang, T.Y. (1986) Anal. Biochem. 157, 323–330.
12 Nozaki, Y., Lasic, D.D., Tanford, C. and Reynolds, J.A. (1982) Science 217, 366–367.
13 Oka, N., Kendall, D.A. and McDonald, R.C. (1982) Biochim. Biophys. Acta 691, 332–340.
14 Gould, G.W., McWhirter, J.M., East, J.M. and Lee, A.G. (1987) Biochim. Biophys. Acta 904, 45–54.
15 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53–56.
16 Eibl, H. and Kovatchev, S. (1981) in Methods Enzymol. 72, 632–639.
17 Jacobson, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152–161.
18 Harlos, K. and Eibl, H.C. (1980) Biochim. Biophys. Acta 601, 113–122.
19 Kolber, M.A. and Haynes, D.H. (1979) J. Membr. Biol. 48, 95–114.
20 Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Biochim. Biophys. Acta 656, 21–51.
21 Madden, T.D. and Cullis, P.R. (1982) Biochim. Biophys. Acta 684, 149–153.
22 Seddon, J.M., Cerec, G. and Marsh, D. (1983) Biochemistry 22, 1280–1289.
23 Yeagle, P.L. and Sen, A. (1986) Biochemistry 25, 7518–7522.
24 Bryszewka, M. and Epard, R.M. (1988) Biochim. Biophys. Acta 943, 485–492.
25 Epand, R.M. and Bryszewska, M. (1988) Biochemistry 27, 8776–8779.
26 Tilcock, C.P.S. and Cullis, P.R. (1981) Biochim. Biophys. acta 641, 189–201.
27 Belly, M.B., Tilcock, C.P.S., Hope, M.J. and Cullis, P.R. (1983) Can. J. Biochem. Cell Biol. 61, 346–352.
28 Wang, C.Y. and Huang, L. (1984) Biochemistry 23, 4409–4416.
29 Demel, R.A., Paltauf, F. and Hauser, H. (1987) Biochemistry 26, 8659–8665.
30 Reynolds, J.A. Nozaki, Y. and Tanford, C. (1983) Anal. Biochem. 130, 471–474.
31 Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2461–2466.