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EFFECT OF ALTERED POLAR HEADGROUP OF PHOSPHATIDYLETHANOLAMINES ON TRANSBILAYER AMINOPHOSPHOLIPID DISTRIBUTION IN SONICATED VESICLES *

AJAY KUMAR and C.M. GUPTA **

Division of Biophysics, Central Drug Research Institute, Lucknow-226001 (India)

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The transbilayer aminophospholipid distributions in small unilamellar vesicles comprising of phosphatidylethanolamine or its analogs (bearing modifications in the polar headgroup) and egg phosphatidylcholine were ascertained using trinitrobenzenesulfonic acid as external membrane probe. These vesicles, containing 10–30 mol% phosphatidylethanolamine or its analogs, were formed by sonication and fractionated by centrifugation. Phosphatidylethanolamine at low concentrations (10 mol%) preferentially localized in the outer monolayer. This preference appeared to be reversed at higher phosphatidylethanolamine concentrations (30 mol%). Unlike this finding, phosphatidylethanolamine bearing ethyl, phenyl and benzyl substituents at the carbon atom adjacent to the amino group distributed mainly in the outer surface irrespective of their concentrations. Similar results were obtained when the phosphate and amino groups were separated by three methylene residues. These observations suggest that the effective polar headgroup volume and/or hydrogen-bonding capacity of phospholipids are the important factors that determine their distribution in small unilamellar vesicles.

Introduction

Presumably, differential functions of the two surfaces of biological membranes dictate the asymmetric distribution of various membrane components in the two bilayer halves. While this asymmetry is absolute for membrane (glyco)proteins [1,2], only partial asymmetry has been observed for membrane phospholipids [3–5]. It is, however, not yet clear as to how this component asymmetry in membranes is generated and maintained.

To understand the intermolecular interactions that might induce formation of asymmetric phospholipid bilayers, small unilamellar vesicles comprised of binary mixtures of phospholipids have been widely used as models for biological membranes [6–13]. It has been reported [6,7] that egg phosphatidylethanolamine in egg phosphatidylcholine (egg PC)/egg phosphatidylethanolamine vesicles prefers to localize in the outer monolayer at low phosphatidylethanolamine (PE) concentrations and in the inner monolayer at higher concentrations. Phosphatidylserine in small unilamellar vesicles containing PC also prefers the inner monolayer [8], at least at low phosphatidylserine concentrations [11]. This behaviour of the aminophospholipids has been rationalised in terms of the charge and packing properties of their polar headgroups [8].

In order to gain a better understanding of the factors which effect the transbilayer arrangement of phospholipids, we have analysed the effect of

* Communication No. 2718 from Central Drug Research Institute, Lucknow, India.

** To whom all correspondence should be addressed.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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altered polar headgroup of PE on its transbilayer distribution in cosonicated mixtures of PE and egg PC. The alterations in the PE headgroup were affected by introducing apolar substituents at the carbon atom adjacent to the amino group (7c–7e, Fig. 1) or by increasing the P-N distance (7b). The transbilayer distributions were determined using trinitrobenzenesulfonic acid (TNBS) as external membrane probe. The results of these studies revealed that phosphatidylethanolamines having larger effective polar headgroup volume prefer to localize in the outer monolayer of small unilamellar vesicles bilayer irrespective of their concentrations.

Materials and Methods

Materials

All the reagents and chemicals used in the study were of the highest purity available. D-Mannitol was obtained from Indian Drugs and Pharmaceuticals Ltd., Hyderabad. Palmitic acid was purchased from Loba Chemie Indoaustranal Co., Bombay. Dicyclohexylcarbodiimide, *N*,*N*-dimethyl-4-aminopyridine, 3-aminopropanol, 2-aminobutanol, 2-amino-2-phenylethanol, 2-amino-3-phenylpropanol, 1,4-diazabicyclo(2,2,2)octane and diisopropylethylamine were bought from Aldrich Chemical Company. [^{14}C]Iodomethane was from Bhabha Atomic Research Centre, Trombay. Sephadex LH-20 (25–100 μm beads) and Bio-Gel A-50m (100–200 μm beads) were bought from Pharmacia Fine Chemicals, Uppsala, Sweden and Bio-Rad Laboratories, Richmond, California, respectively. Silica gel (60–120 mesh) and Dowex 50W × 8 were obtained from Sisco Research Laboratories, Bombay and Baker Chemical Company, Phillipsburg, NJ, respectively. Silica gel 60F-254 (0.25 mm thickness, 20 × 20 cm) pre-coated plastic sheets were purchased from E. Merck, Darmstadt, F.R.G.

General methods

Purity of various compounds was routinely checked by TLC using Silica gel G-60 plates. For phosphatidylcholine and phosphatidylethanolamine (7a), TLC plates were developed in chloroform/methanol/water (65 : 25 : 4, v/v) and the spots were identified after staining the plate with iodine vapors followed by molybdenum-blue spray [14] while in case of modified phosphatidylethanolamines (7b–7e, Fig. 1), chloroform/methanol/water (70 : 15 : 2, v/v) was used as TLC solvent system. Purification of phospholipids was carried out on Sephadex LH-20 column (2.5 × 100 cm) using chloroform/methanol (1 : 1, v/v) as the eluant [15] and in some instances by preparative TLC. Total phosphorus was estimated by the method of Ames and Dubin [16].

All the compounds were characterized by infrared and NMR spectroscopy. The infrared spectra were recorded in Perkin-Elmer-IR-177 Grating infrared spectrophotometer and NMR spectra were recorded in a Perkin-Elmer R-32 NMR spectrophotometer. Resonances (δ) in the NMR spectra is given in ppm downfield from tetramethylsilane. The assay of radioactive isotopes was carried out in a Packard Tricarb 3330 liquid scintillation spectrometer with 2,5-diphenyloxazole (4.0 g), 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.2 g), 2-methoxyethanol (500 ml) and toluene (500 ml) as the scintillator.

Preparations of phospholipids

Egg PC was isolated according to the published procedure [17] and egg [*Me*-^{14}C]PC (25 μCi/μmol) was prepared according to Gupta and Bali [18]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (7a, Fig. 1) and its analogs (7b–7e, Fig. 1) were prepared from D-mannitol. A brief account of the

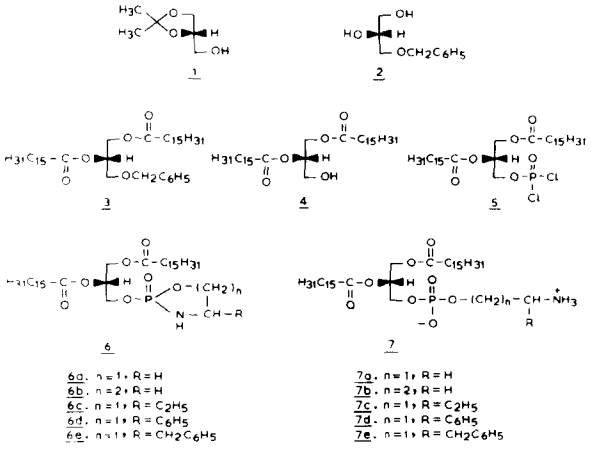

Fig. 1. Preparation of modified phosphatidylethanolamines (7a–7e).

procedure used is given below.

1,2-Isopropylidene-*sn*-glycerol (1) was prepared from D-mannitol essentially according to the published method [19]. Yield 65–75%, b.p. 70–75°C/2 mm, $[\alpha]_D^{20}$ 15.05° at 589 nm (neat). ν_{max} (KBr): 3400 (O-H) and 1060 cm^{-1} (C-O-C); δ 4.1–3.8 (m, 1 H, CH), 3.6 (d, $J = 6$ Hz, 2 H, CH_2-O), 3.45 (d, $J = 6$ Hz, 2 H, CH_2-O), 1.3 (s, 3 H, CH_3), 1.2 (s, 3 H, CH_3). *sn*-Glycerol-3-benzyl ether (2) was prepared from 1 essentially by the procedure of Howe and Malkin [20]. Yield 75–83%, b.p. 145–150°C/2mm. ν_{max} (KBr): 3400 (O-H) and 1100 cm^{-1} (C-O-C); δ 7.15 (s, 5 H, C_6H_5), 4.35 (s, 2 H, OCH_2-C_6H_5), 3.9–3.6 (m, 1 H, CH-O), 3.45 (d, $J = 6$ Hz, 2 H, CH_2-O), 3.35 (d, $J = 6$ Hz, 2 H, CH_2-O).

The benzyl glycerol 2 was reacted in chloroform with palmitic anhydride in the presence of trace amounts of *N,N*-dimethyl-4-aminopyridine [15] to give 1,2-dipalmitoyl-*sn*-glycerol-3-benzyl ether (3). The product was crystallized from ethanol. Yield 66–78%, m.p. 44–45°C. ν_{max} (KBr): 1750 (C = O) and 1160 cm^{-1} (C-O-C); δ 7.2 (s, 5 H, C_6H_5), 5.15–4.9 (m, 1 H, C-H), 4.4 (s, 2 H, $OCH_2C_6H_5$), 4.05 (d, $J = 6$ Hz, 2 H, CH_2-O), 3.5 (d, $J = 6$ Hz, 2 H, CH_2-O).

The diacyl benzyl glycerol 3 was debenzylated according to the known method [20] to give 1,2-dipalmitoyl-*sn*-glycerol (4). Yield 76–85%, m.p. 63–65°C. ν_{max} (KBr): 3500 (O-H) and 1740 cm^{-1} (C = O); δ 5.0–4.7 (m, 1 H, CH), 4.1 (d, $J = 6$ Hz, 2 H, CH_2-O), 3.5 (bs, 2 H, CH_2-O).

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (7a), 1,2-dipalmitoyl-*sn*-glycero-3-phosphopropanolamine (7b), 1,2-dipalmitoyl-*sn*-glycero-3-phospho(α-ethyl)ethanolamine (7c), 1,2-dipalmitoyl-*sn*-glycero-3-phospho(α-phenyl)-ethanolamine (7d) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho(α-benzyl)ethanolamine (7e) were prepared from 4 by slight modifications of the published procedure [21]. It was observed that if conversion of 4 into 1,2-dipalmitoyl-*sn*-glycero-3-phosphoric acid dichloride (5) is carried out at the reported [21] temperature (+5°C), a large amount of polymeric material is formed which renders it difficult to isolate 5 in pure form. However, this reaction at temperatures close to −10°C yielded 5 in almost pure form. Similarly, to avoid the formation of polymeric materials, the reactions of the phosphoryl dichloride 5 with aminoalcohols were done at low temperature (approx. −10°C) rather than at the reported [21] temperature (+10°C). Finally, incomplete hydrolyses of the cyclic phosphates (6a–6e, Fig. 1) occurred when these were carried out in the published conditions [21]. For complete hydrolysis, it was found necessary to carry out these reactions at 40–45°C for 12 h. PE analogs (7a–7e) so obtained were purified to homogeneity using Sephadex LH-20 and preparative thin-layer chromatography.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (7a) was characterized by its co-chromatography with the pure sample of this phospholipid, obtained from Sigma Chemical Company, on TLC plates. The compounds 7b–7e were characterized by IR, NMR and C, H and N analyses. The optical purity of the glycerol backbone in 7a–7e was established by their hydrolyses with phospholipase A_2. Yield (7a–7e) 40–55%.

7b. ν_{max} (KBr): 1750 cm^{-1} (C = O); δ 5.3–5.0 (m, 1 H, C-H), 4.4–5.1 (m, 4 H, CH_2-OP), 4.1–3.9 (m, 2 H, CH_2-O-CO), 3.2–3.0 (m, 2 H, CH_2-N). Analysis: Calcd. for $C_{38}H_{76}NO_8P$: C, 64.68; H, 10.78; N, 1.99. Found: C, 64.80; H, 10.31; N, 2.21%.

7c. ν_{max} (KBr): 1750 cm^{-1} (C = O); δ 5.3–5.0 (m, 1 H, C-H), 4.3–4.0 (m, 4 H, CH_2-OP), 4.0–3.7 (m, 2 H, CH_2-O-CO), 3.6–3.4 (m, 1 H, CH-N). Analysis: Calcd. for $C_{39}H_{78}NO_8P$: C, 66.65; H, 10.80; N, 1.95. Found: C, 66.09; H, 10.85; N, 2.14%.

7d. ν_{max} (KBr): 1740 cm^{-1} (C = O); δ 7.15 (s, 5 H, C_6H_5), 5.3–5.0 (m, 1 H, C-H); 4.4–4.0 (m, 4 H, CH_2-OP), 4.0–3.6 (m, 3 H, CH_2-OCO and CH-N). Analysis: Calcd. for $C_{43}H_{78}NO_8P.4H_2O$: C, 61.50; H, 10.25; N, 1.67. Found: C, 61.70; H, 10.30; N, 1.76%.

7e. ν_{max} (KBr): 1740 cm^{-1} (C = O); δ 7.15 (s, 5 H, C_6H_5), 5.0–4.7 (m, 1 H, C-H), 4.3–2.8 (m, 9 H, CH_2-OCO, CH_2-O-P and CH-$CH_2C_6H_5$). Analysis: Calcd. for $C_{44}H_{80}NO_8P.H_2O$: C, 66.08; H, 10.26; N, 1.76. Found: C, 66.25; H, 10.28; N, 1.69%.

Preparation of small unilamellar vesicles

A solution of egg PC, traces of ^{14}C-labeled egg PC and varying mol fractions (10, 20 and 30

mol%) of one of the compounds 7a–7e in chloroform/methanol (1 : 1, v/v) mixture was evaporated in a glass tube under a slow jet of N_2, resulting in the formation of a thin lipid film on the wall of the tube. Final traces of the solvents were removed by leaving the tube in vacuo for 3–4 h. The lipid mixture was dispersed in 10 mM phosphate buffer (pH 8.5) so as to achieve a concentration of approx. 6 μmol of phosphatidylethanolamines/ml buffer. It was vortexed for 5–10 min at 40°C. The lipid dispersion so obtained was carefully transferred to a cuvette and sonicated at 0°C under N_2 atmosphere using a probe-type sonicator (MSE 150 W, 20 kH_z) to give an optically clear suspension (35–45 min). Degradation of phospholipids during sonication was routinely checked by TLC. No appreciable amounts of lysophospholipids were detected in the samples. The sonicated preparations were centrifuged at 105 000 × *g* (Ti-50 fixed angle rotor) for 60 min at 10°C to affect the removal of titanium particles as well as poorly dispersed lipids. Only the top two-third of the total supernatant volume in the centrifuge tube was used in the study.

The size distribution of vesicles was analysed by chromatography of the vesicle preparations on Bio-Gel A-50m column. A downward flowing colume (40 × 1.2 cm) of Bio-Gel A-50m maintained at constant hydrostatic pressure was equilibrated with 10 mM Tris-HCl buffer (pH 7.2). A measured aliquot (0.4 ml) of the vesicle preparation was applied to the column and eluted with the same buffer at 6.5 ml/h. The void volume of the column was determined by Blue Dextran 2000.

The percentage incorporations of PE analogs in vesicles were estimated as follows: The liposome-rich fractions from Bio-Gel A-50m column were pooled together and then lipids were extracted by Bligh-Dyer extraction procedure [22]. The lipid mixture was chromatographed over silica gel 60F-254 pre-coated plastic sheets (10 × 4 cm). The spots corresponding to egg PC and phosphatidylethanolamine were removed and eluted with chloroform/methanol (1 : 1, v/v) mixture. The total phosphorus in each spot was determined as described [16]. The amounts of PE incorporated in vesicles in each case were > 90%.

Determination of phosphatidylethanolamine distribution

PE distribution in the two surfaces of small unilamellar vesicles was ascertained essentially by the published procedure [23]. To an aliquot (40 μl) of vesicle preparations, containing not more than 0.25 μmol of PE, were added 10 mM phosphate buffer (pH 8.5; 560 μl) and 0.8 M $NaHCO_3$ (pH 8.5; 200 μl). After vortexing the mixture, a 20-μl aliquot of 1.5% TNBS solution in 0.8 M $NaHCO_3$ (pH 8.5) was added and the mixture incubated at 20 ± 2°C in dark for different time intervals (10, 20, 30, 60, 120 and 180 min). The reaction was terminated by adding 1.2% Triton X-100 (400 μl) in 1.5 M HCl. Absorbance was read within 1 h at 410 nm after termination of the reaction. The total PE content in vesicles was determined as follows: an aliquot of vesicle preparation (40 μl), containing not more than 0.25 μmol PE, was diluted to a final volume of 0.6 ml with buffer. To this was added 200 μl of 1.6% Triton X-100 in 0.8 M $NaHCO_3$ (pH 8.5). The sample was mixed. A 20-μl aliquot of the 1.5% TNBS solution was added. The sample was vortexed and incubated in dark at 20 ± 2°C for the same time intervals as above. After incubation, 0.4% Triton X-100 in 1.5 M HCl (400 μl) was added. The sample was vortexed and stored in dark. The absorbance at 410 nm was read within 1 h of acidification. The absorbance at 410 nm was linear with concentration to at least 0.8 absorbance units.

Results and Discussion

The structural changes that were introduced in the polar headgroup of 7a to obtain 7b–7e were based on the consideration that the change should not appreciably alter the charge properties of the prototype molecule. Therefore, only apolar residues were used to affect the alterations in the effective polar headgroup volume (or length) of 7a. Use of three or four carbon atom long aliphatic hydrocarbon substituents was avoided due to the reason that such groups may significantly change the solubility of the phospholipid polar headgroup in water and consequently the headgroup conformation. The fact that aromatic hydrocarbons are lesser hydrophobic than the corresponding aliphatic hydrocarbons because of stronger van

der Waals attraction between water molecules and their π-electrons [24], it was presumed that phenyl or benzyl substituents would prove better than *n*-propyl or *n*-butyl residues.

Varying mol fractions (10, 20 and 30%) of 7a or one of its analogs 7b–7e were mixed with egg PC and the dried lipid mixture after dispersing in buffer was sonicated. The sonicated preparations were fractionated by centrifugation. Only those vesicles which were present in the top 70% of the total supernatant volume in the centrifuge tube were used in the study. A portion of vesicles was chromatographed over Bio-Gel A-50m column to analyse their size distribution. The typical elution profiles of vesicles from the column are shown in Figs. 2 (10 mol% PE) and 3 (30 mol% PE). Most of the vesicles eluted much after the void volume (V_0) of the column and were therefore considered as small and unilamellar [25]. As indicated by the elution patterns (Figs. 2 and 3), replacement of 7a in vesicles by any one of its analogs 7b–7e does not seem to affect significantly the size distributions of vesicles. The outer diameter of the vesicles, as determined by negative staining electron microscopy, was approx. 25–60 nm. Percentage incorporations of 7a or its analogs 7b–7e in vesicles were also determined. In each case the incorporations were > 90%.

Transbilayer PE distribution in vesicles was determined by labeling the vesicles with TNBS at 20 ± 2°C for different periods of time. After each time period, the reactions were terminated by lowering the pH of the reaction medium from 8.5 to approx. 3. Yellow color in these reaction mixtures was measured at 410 nm [23]. Parallel experiments in identical conditions were also carried out on lysed vesicles. The amounts of PE labeled, as judged by absorbance at 410 nm, in lysed vesicles were invariably higher than that in the intact vesicles (Figs. 4 and 5). A continuous rise in the amounts of labeled PE was observed during the first 30 min. These amounts after this period remained almost constant upto 3 h. It was inferred from these experiments that reaction of TNBS with intact vesicles results in modification of only those PE molecules which are localized in the external monolayer of small unilamellar vesicles bilayer. Using the 3 h absorbance values of lysed vesicles as the values for the total amount of

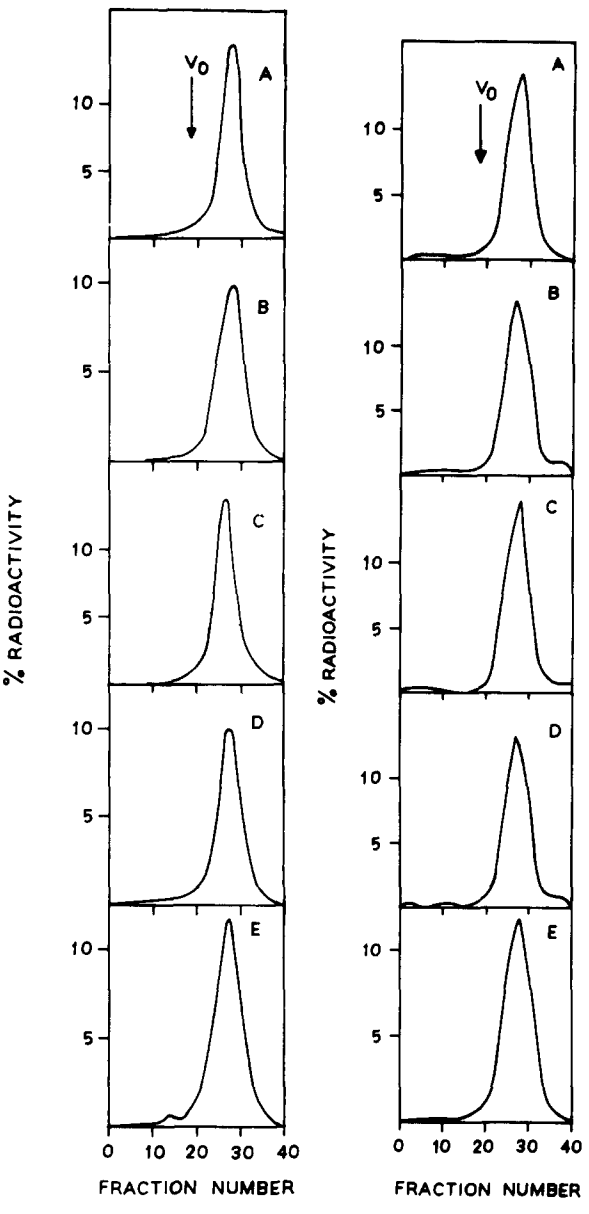

Fig. 2. (Left). Bio-Gel A-50m flow-through patterns of vesicles prepared from egg phosphatidylcholine (egg PC), traces of ^{14}C-labeled egg PC and phosphatidylethanolamine (10 mol%). A, 7b/egg PC; B, 7c/egg PC; C, 7d/egg PC; D, 7e/egg PC; E, 7a/egg PC.

Fig. 3. (Right). Bio-Gel A-50m flow-through patterns of vesicles prepared from egg phosphatidylcholine (egg PC), traces of ^{14}C-labeled egg PC and phosphatidylethanolamine (30 mol%). A, 7b/egg PC; B, 7c/egg PC; C, 7d/egg PC; D, 7e/egg PC; E, 7a/egg PC.

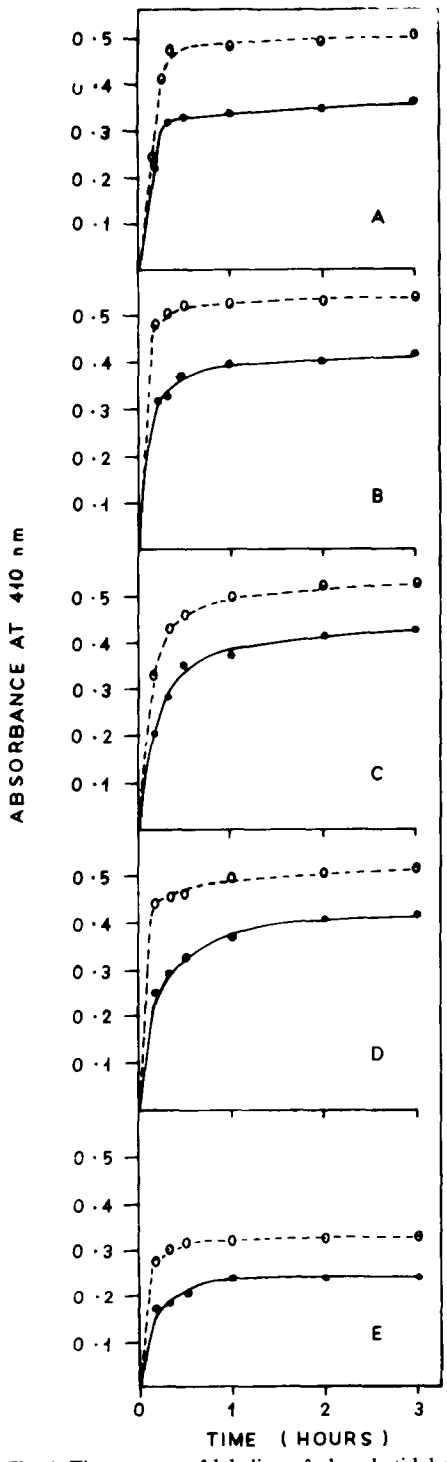

Fig. 4. Time-course of labeling of phosphatidylethanolamine in phosphatidylethanolamine (10 mol%)/egg phosphatidylcholine (egg PC) vesicles with trinitrobenzenesulfonic acid. A, 7b/egg PC; B, 7c/egg PC; C, 7d/egg PC; D, 7e/egg PC; E, 7a/egg PC. ●———●, intact vesicles; O— — —O, lysed vesicles.

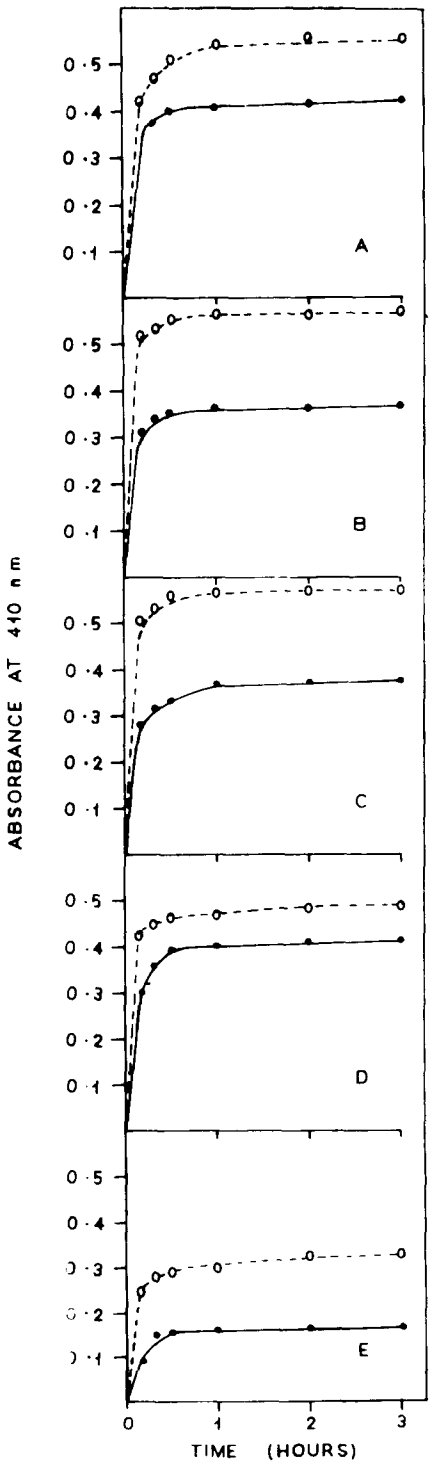

Fig. 5. Time-course of labeling of phosphatidylethanolamine in phosphatidylethanolamine (30 mol%)/egg phosphatidylcholine (egg PC) vesicles with trinitrobenzenesulfonic acid. A, 7b/egg PC; B, 7c/egg PC; C, 7d/egg PC; D, 7e/egg PC; E, 7a/egg PC. ●———●, intact vesicles; O— — —O, lysed vesicles.

vesicle PE, the amounts of external PE in each vesicle preparation were calculated. A summary of the results is given in Table I.

As may be seen in Table I, the amounts of the external 7a decrease with an increase in mole fractions of this phospholipid in vesicles. These results are in accordance with the earlier observations of Litman [6,7]. However, no such decrease was observed when 7a was replaced by 7b–7e in vesicles. These results, therefore, strongly suggest that an increase in the polar headgroup volume (or length) of 7a renders it insensitive to undergo concentration-dependent changes in its localization in small unilamellar vesicles.

Phospholipids having widely differing thermal phase transition temperatures (T_m) form separate phases in bilayers at temperatures below the T_m of the higher melting component [26,27]. The two phospholipid components employed in the present study, i.e. egg PC and 7a, are known to undergo thermal phase transitions at markedly different temperatures [26,27] and thus are not likely to mix with each other at 20°C. Inspite of the fact that 7a and egg PC must be forming two separate phases in small unilamellar vesicles bilayers at 20°C, the amounts of the external 7a in vesicles were similar to that observed for egg PE in egg PE/egg PC vesicles [6,7]. This finding suggests that in small unilamellar vesicles comprising of binary mixtures of phospholipids, the inside-outside distributions of the phospholipid components are probably not influenced by their mutual miscibility.

TABLE I

LOCALIZATION OF PHOSPHATIDYLETHANOLAMINES IN THE OUTER SURFACE OF SMALL UNILAMELLAR VESICLES (SUV)

Values shown are mean of three determinations ±S.D. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

SUV	Molar ratio PE:PC	Outer PE/ total PE (%)
7a/egg PC	1:9	73.89±3.79
	2:8	65.58±0.75
	3:7	48.21±1.51
7b/egg PC	1:9	73.49±1.36
	2:8	68.87±1.77
	3:7	64.62±1.86
7c/egg PC	1:9	76.05±1.69
	2:8	75.02±2.56
	3:7	75.29±3.09
7d/egg PC	1:9	78.20±1.23
	2:8	77.73±1.43
	3:7	68.26±0.66
7e/egg PC	1:9	77.69±2.89
	2:8	73.70±1.37
	3:7	69.07±3.03

Several studies have shown that the transbilayer movement (flip-flop) of phospholipids in model membrane systems is a very slow process [3]. However, this process may be accelerated under some conditions [5]. The fact that there was no time dependent increase in the amounts of labeled 7a–7e in vesicles, it is concluded that there were no rapid transmembrane movements of 7a–7e from the inner to the outer leaflet of the small unilamellar vesicles bilayer.

The fraction of phospholipid molecules in the outer surface of small vesicles is about two-third and this fraction decreases with an increase in the vesicle size [28]. This suggests that the differences observed between the amounts of the external 7a and the external 7b–7e in the present study could be due to variations in the sizes of vesicles. But this is not likely because the elution profiles of various vesicle preparations, containing a specified mol fraction of PE, were similar from Bio-Gel A-50m column. Therefore, the differences seen between the amounts of the external 7a and the external 7b–7e must be a consequence of alterations introduced in the polar headgroup of 7a.

The charge in the phospholipid headgroup has been considered of primary importance in determining lipid distribution in mixed bilayers [8,9,12]. As the charge properties of the polar headgroups of the PE analogs (7b–7e) should not be different from that of 7a, it is expected that these phospholipids may have their transbilayer distributions similar to that of the parent molecule. Since, these distributions were markedly different, it is inferred that the polar headgroup charge is not the main determinant of the phospholipid distribution in small unilamellar vesicles.

Israelachvili and co-workers have developed a theory to explain phospholipid distributions in vesicles [29,30]. These workers have claimed that the transbilayer distribution of phospholipids could

be predicted by knowing the optimal hydrocarbon-water interfacial areas, the hydrocarbon chain volumes and critical lengths, and the effective headgroup length parameters D, of the components. The fact that all these four structural parameters of 7c–7e are expected to be similar to that of 7a, some additional factors like intermolecular hydrogen bond forming capacity and/or the effective polar headgroup volume of phospholipids must be taken into consideration.

The area occupied by each phospholipid headgroup in the inner surface of small unilamellar vesicles is similar to that in planar bilayers whereas it is larger at the outer surface [31], suggesting that the curvature of small vesicles may in some cases enforce such transbilayer arrangements which otherwise are not favored. This is consistent with the observations that the transbilayer distribution of PE in small vesicles varies with its concentration but the same lipid in larger size vesicles (outer diameter 60–80 nm) at its varying concentrations prefers the random distribution [23]. Besides this, the distributions of phosphatidic acid [10] and phosphatidylglycerol [13] are random even in small vesicles. These findings thus suggest that the concentration dependent changes seen by us and also by others [6,7] in the distributions of PE must have resulted from some such unique property of this phospholipid which becomes effective only in the highly curved systems. It is tempting to speculate here that this unique property of PE is its capacity to form intermolecular hydrogen bonds.

Hydrogen bonds are highly directional in nature and their effective formation requires a specific orientation of the donor and the acceptor centers [32]. Such an orientation might be adversely affected by the introduction of substituents at the carbon atom adjacent to the amino group of 7a and also by increasing the P-N distance, which inturn may result in differences between the behaviors of 7a and its analogs. Therefore, it is concluded from these studies that the effective polar headgroup volume and/or hydrogen bonding capacity, rather than the polar headgroup charge and the effective headgroup length, are the main parameters which control the transbilayer distributions of phospholipids in small unilamellar vesicles. The term effective headgroup volume used here includes the volume of the hydration shell which forms around the headgroup in water.

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