of the wavelength spectrum (unpublished results).

As the scattering contribution to the signal disappears, the structure either disappears also or changes in organization such that it becomes nonchiral, or the parameters become much larger or much smaller than the range of wavelengths covered by the spectrum.

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Transbilayer Phosphatidylcholine Distributions in Small Unilamellar Sphingomyelin–Phosphatidylcholine Vesicles: Effect of Altered Polar Head Group†

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ABSTRACT: The effect of the altered polar head group of phosphatidylcholine (PC) on its transbilayer distributions in small unilamellar vesicles containing sphingomyelin (SM) was ascertained with phospholipase A_2 as the external membrane probe. These vesicles were formed by sonication and fractionated by centrifugation. The vesicle size was determined by gel-permeation chromatography and solute entrapment. Experiments were done to confirm that phospholipase A_2 treatments did not induce fusion, lyse the vesicles, or cause PC to migrate across the vesicle bilayer. The complete degradation of external PC in intact vesicles was assured by carrying out the enzyme reactions in the absence as well as in the presence of 9.2×10^{-5} M bovine serum albumin. In small vesicles comprised of SM and 30 mol % 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), DPPC preferentially distributed in the inner monolayer. This preference of DPPC in these vesicles disappeared upon introducing one C_2H_5 group at the carbon atom adjacent to the quaternary ammonium residue in its polar head group and was reversed when the C_2H_5 group was replaced by C_6H_5 and $C_6H_5CH_2$ substituents or when the P–N distance was increased. These results indicate that the effective polar head-group volume is an important factor in determining the phospholipid distributions across the small vesicle bilayer.

Asymmetric distributions of various membrane components across the membrane bilayer are perhaps essential for differential functioning of the two sides of biological membranes. There is now much evidence to suggest that various membrane constituents are asymmetrically localized in the two leaflets of the membrane bilayer (Op den Kamp, 1979). While this asymmetry is absolute for membrane (glyco) proteins, only partial asymmetry is observed for membrane phospholipids.

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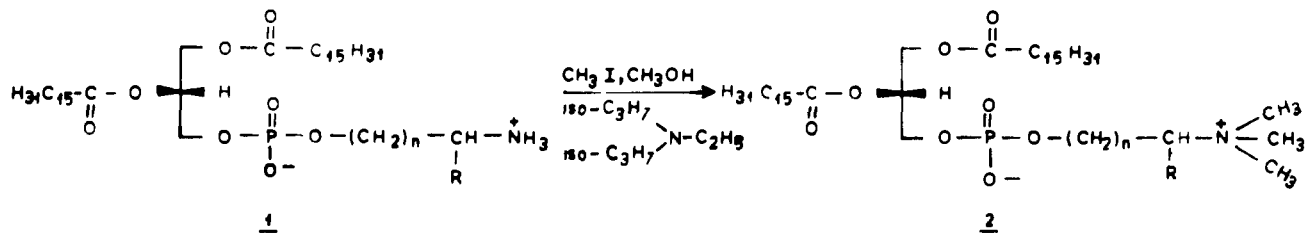

1

2

1a. n = 1 , R = H
1b. n = 2 , R = H
1c. n = 1 , R = C_2H_5
1d. n = 1 , R = C_6H_5
1e. n = 1 , R = $CH_2C_6H_5$

2a. n = 1 , R = H
2b. n = 2 , R = H
2c. n = 1 , R = C_2H_5
2d. n = 1 , R = C_6H_5
2e. n = 1 , R = $CH_2C_6H_5$

FIGURE 1: Preparation of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (**2a**) and its analogues (**2b–e**) from the corresponding phosphatidylethanolamines.

Numerous studies have been conducted in small unilamellar mixed-phospholipid vesicles to understand the factors that induce formation of asymmetric phospholipid bilayers. It has been shown that sphingomyelin (SM)[1] in small unilamellar vesicles (SUV) consisting of SM and phosphatidylcholine (PC) preferentially distributes in the outer surface of the SUV bilayer (Berden et al., 1974, 1975; Castellino, 1978) whereas phosphatidylserine (PS) in PC–PS vesicles prefers the inner monolayer (Berden et al., 1975), at least at low PS concentrations (Barsukov et al., 1980). Phosphatidylethanolamine (PE) in curved vesicles comprised of PC and PE (>10 mol %) also prefers the inner monolayer (Litman, 1973, 1974). But this preference of PE in these vesicles is reversed on increasing its polar head-group volume (Kumar & Gupta, 1983).

To further investigate the role of phospholipid head-group volume in determining the phospholipid distributions across the SUV bilayer, we have studied the effect of altered polar head group of PC on its transbilayer distributions in cosonicated mixtures of PC and SM. The alterations in the PC head group were affected by introducing apolar substituents at the carbon atom adjacent to the onium group (**2c–e**, Figure 1) or by increasing the P–N distance (**2b**). The transbilayer distributions were ascertained with *Naja naja* phospholipase A_2 as the external membrane probe. The experiments were carefully controlled to assure that the enzyme treatments did not induce fusion, lyse the vesicles, or cause the phospholipid to migrate across the bilayer. Results of these studies indicate that phospholipids having a larger head-group surface volume invariably distribute preferentially in the outer monolayer.

Materials and Methods

Materials. All the reagents and chemicals used were of the highest purity available. 6-Carboxyfluorescein (6-CF) was from Eastman Kodak Co. Phospholipase A_2 was purified from *Naja naja* snake venom (Haffkine Institute, Bombay) by the method of Blecher (1966) to a protein concentration of approximately 1 mg/mL. [^{14}C]Glucose and iodo[^{14}C]methane were purchased from Bhabha Atomic Research Centre, Trombay. Diisopropylethylamine and 1,4-diazabicyclo(2.2.2)octane were bought from Aldrich Chemical Co. Sephadex LH-20 (25–100-μm beads) and Sephadex G-50 (20–80-μm beads) were procured from Pharmacia Fine Chemicals. Bio-Gel A-50m was from Bio-Rad Laboratories. Silica gel (60–120 mesh, activity 2–3) and precoated silica gel 60F-254 thin-layer chromatography (TLC) plates were bought from Sisco Research Laboratory, Bombay, and E. Merck, respectively. Southern bean mosaic virus (SBMV) was a kind gift from Professor O. P. Sehgal, University of Missouri, Columbia, MO.

General Methods. Purity of various compounds was routinely checked by TLC on silica gel G-60 plates. For SM, **2a** and **2b** TLC plates were developed in chloroform/methanol/water (65:25:4 v/v) while in the cases of **2c–e** chloroform/methanol/water (70:20:2 v/v) was used as the TLC solvent system. Sephadex LH-20 chromatography was done over a 2.5 × 100 cm column with chloroform/methanol (1:1 v/v) as the eluant. The rate of elution was about 60 mL/h. One-milliliter fractions were collected. Each fraction was monitored by TLC. Spots on TLC plates were visualized by staining the plate with iodine vapor followed by molybdenum blue spray (Goswami & Frey, 1971). All the phospholipid samples used in this study exhibited single spots on TLC plates. Total phosphorus was estimated by the method of Ames & Dubin (1960).

All the **2a** analogues (**2b–e**) were characterized by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopies. The IR spectra were recorded in a Perkin-Elmer IR-177 grating spectrophotometer, and NMR spectra were recorded in a Perkin-Elmer R-32 NMR spectrophotometer. Resonance (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 diphenyloxazole (4.0 g), 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (0.2 g), 2-methoxyethanol (500 mL), and toluene (500 mL) as the scintillator. 6-CF was measured on an Aminco SPF-500 fluorometer using excitation and emission wavelengths of 490 and 520 nm, respectively.

Phospholipids. SM was isolated from outdated human blood. Briefly, after erythrocytes were separated from white blood cells, red cell lipids were extracted (Rose & Oklander, 1965). SM from the crude lipid extract was isolated and purified as described earlier (Kumar & Gupta, 1984).

[1] Abbreviations: SUV, small unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 6-CF, 6-carboxyfluorescein; TLC, thin-layer chromatography; IR, infrared; NMR, nuclear magnetic resonance; s, singlet; d, doublet; m, multiplet; SBMV, southern bean mosaic virus; γ-Ig, γ-immunoglobulin; V_0, void volume; V_i, internal volume; T_m, melting phase transition temperature; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[*methyl*-^{14}C]SM (15 μCi/μmol) was prepared from SM following the demethylation/remethylation procedure of Gupta & Bali (1981). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained by acylating 1-palmitoyl-*sn*-glycero-3-phosphocholine with oleic anhydride (Gupta et al., 1977). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine and its analogues (**1b–e**) were synthesized following the published method (Kumar & Gupta, 1983). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (**2a**), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*,*N*,*N*-trimethylpropanolamine (**2b**), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*,*N*,*N*-trimethyl-α-ethylethanolamine (**2c**), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*,*N*,*N*-trimethyl-α-phenylethanolamine (**2d**), and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*,*N*,*N*-trimethyl-α-benzylethanolamine (**2e**) were prepared by treating the corresponding phosphatidylethanolamines (**1a–e**) with iodomethane/diisopropylethylamine in methanol (Gupta & Bali, 1981). Typically, 160 mg of either of the phosphatidylethanolamines, **1a–e**, was suspended in anhydrous methanol (5.0 mL), and to it were added diisopropylethylamine (0.5 mL) and iodomethane (1.5 mL). The reaction flask was tightly stoppered, and the mixture was stirred at room temperature (25–35 °C) in the dark for 20–30 h. Progress of the reaction was monitored by TLC. After the reaction was complete, the solvent was removed under reduced pressure. The residue was chromatographed over silica gel column with increasing amounts of methanol in chloroform as the eluant. The pure fractions were pooled together, and after the solvents were removed, the residue was rechromatographed over Sephadex LH-20 to afford pure samples of **2a–e**.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (**2a**) was characterized by its cochromatography and superimposable IR and NMR with those of the pure sample of this phospholipid, obtained from Sigma Chemical Co. The compounds **2b–e** were characterized by IR, NMR, and C, H, and N analyses. The optical purity of the glycerol backbone in **2a–e** was established by their hydrolyses with phospholipase A_2, yield (**2a–e**) 50–65%.

Analysis of **2b** showed the following: IR ν_{max} (KBr) 1750 (C=O) cm^{-1}; NMR δ 5.2–4.9 (m, 1 H, CH), 4.7–4.1 (m, 4 H, CH_2–O–P), 4.1–3.7 (m, 2 H, CH_2–O–CO), 3.6–3.4 (m, 2 H, CH_2–N), 3.3 [s, 9 H, $N(CH_3)_3$], and 2.3–2.0 (m, 4 H, CH_2–COO). Anal. Calcd for $C_{41}H_{82}NO_8P$: C, 65.86; H, 10.97; N, 1.87. Found: C, 65.45; H, 10.85; N, 2.12.

Analysis of **2c** showed the following: IR ν_{max} (KBr) 1740 (C=O) cm^{-1}; NMR δ 5.3–5.0 (m, 1 H, CH), 4.9–4.0 (m, 4 H, CH_2–O–P), 4.0–3.6 (m, 2 H, CH_2–O–CO), 3.6–3.4 (m, 1 H, CH–N), 3.2 [s, 9 H, $N(CH_3)_3$], and 2.5–2.0 (m, 4 H, CH_2–COO). Anal. Calcd for $C_{42}H_{84}NO_8P{\cdot}H_2O$: C, 64.69; H, 11.04; N, 1.80. Found: C, 64.39; H, 10.85; N, 1.72.

Analysis of **2d** showed the following: IR ν_{max} (KBr) 1740 (C=O) cm^{-1}; NMR δ 7.3 (s, 5 H, C_6H_5), 5.5–5.0 (m, 1 H, CH), 4.7–4.5 (m, 1 H, CH–N), 4.5–4.1 (m, 4 H, CH_2–O–P), 4.1–3.8 (m, 2 H, CH_2–O–CO), 3.4–3.2 [s, 9 H, $N(CH_3)_3$], and 2.3–1.9 (m, 4 H, CH_2–COO). Anal. Calcd for $C_{46}H_{84}NO_8P{\cdot}2H_2O$: C, 66.75; H, 10.39; N, 1.66. Found: C, 67.05; H, 10.29; N, 1.54.

Analysis of **2e** showed the following: IR ν_{max} (KBr) 1740 (C=O) cm^{-1}; NMR δ 7.4 (s, 5 H, C_6H_5), 5.0–4.7 (m, 1 H, CH), 4.5–3.2 (m, 9 H, CH_2–O–P, CH_2–OCO, C*H*–CH_2–C_6H_5), and 3.35 [s, 9 H, $N(CH_3)_3$]. Anal. Calcd for $C_{47}H_{86}NO_8P$: C, 68.53; H, 10.45; N, 1.70. Found: C, 68.85; H, 10.60; N, 1.86.

Small Unilamellar Vesicles. A solution of SM, traces of ^{14}C-labeled SM, and about 30 mol % of one of the compounds **2a–e** 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 dried lipid mixture was dispersed in Tris-buffered saline (10 mM Tris containing 150 mM NaCl, pH 7.4) so as to achieve a concentration of about 1.25 μmol of phosphatidylcholines/mL of buffer. It was vortexed for 5–10 min at room temperature (25–35 °C). The lipid dispersion so obtained was carefully transferred to a water-jacketed cuvette and sonicated (40 °C) under N_2 in a probe-type sonicator (Heat Systems, W-220F) to give an optically clear suspension (35–50 min). The sonicated preparations were centrifuged at 105000*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 vesicles found in the top two-thirds of the supernatant were used in the study.

Vesicle Size Determination. The vesicle size was determined by analytical molecular sieve chromatography on Bio-Gel A-50m at 22 ± 2 °C. A downward flowing column (1 × 50 cm) of Bio-Gel A-50m maintained at constant hydrostatic pressure was equilibrated with Tris-buffered saline. A measured aliquot (0.5 mL) of each vesicle preparation was applied to the column and eluted with the same buffer at 8 mL/h. One-milliliter fractions were collected and analyzed by measuring total phosphorus or ^{14}C.

The Stokes radii of vesicles eluted from the column were calculated according to Ackers (1967). The void volume (V_0) and internal volume (V_i) were determined by using Blue Dextran 2000 (or multilamellar vesicles) and glycylglycine as the markers, respectively. SBMV (Stokes radius 160 Å) and γ-Ig (Stokes radius 52 Å) were used to calibrate the column.

Entrapment Capacity of Vesicles. For determining the entrapment capacity of vesicles, SUV were prepared from SM and 30 mol % of one of the phosphatidylcholines **2a–e** in Tris buffer containing [^{14}C]glucose (about 5 μCi), by sonication. The vesicle preparations were fractionated by ultracentrifugation as described above. After the total amounts of ^{14}C and lipid P associated with the fractionated preparation were measured, a known volume of this preparation was carefully chromatographed on a Sephadex G-50 column (1.4 × 40 cm) for effecting separations between the entrapped and free glucose. The column was eluted with the same buffer at 22 mL/h (4 °C). Fractions (0.5 mL each) were analyzed for ^{14}C and phosphorus. Vesicles were invariably recovered in the void volume of the column. Recoveries of ^{14}C and lipid P from the column were over 95%.

Vesicle Phospholipid Hydrolysis by Phospholipase A_2. Vesicles (2–4 μmol of lipid P) were incubated with 20 μg of phospholipase A_2 in Tris-buffered saline (10 mM Tris containing 150 mM NaCl and 10 mM $CaCl_2$, pH 8.5) in a total volume of 1.0 mL for 3 h at 25–30 °C with or without bovine serum albumin (9.2×10^{-5} M) or 2% methanol in diethyl ether (1 mL). The reactions were terminated by addition of EDTA (100 mM, 1.0 mL). The lipids were extracted, and the intact and hydrolyzed phospholipids were separated by TLC on E. Merck silica gel plates. The plates were developed as described under General Methods. Spots were removed and eluted several times with a chloroform/methanol (1:1 v/v) mixture. Total phosphorus present in each spot was determined according to Ames & Dubin (1960). The recoveries of phospholipids from silica gel were at least 90–96%.

Kinetics of Phospholipase A_2 Hydrolysis. Kinetics of PC hydrolysis by phopholipase A_2 was studied in small vesicles comprised of SM and **2a** (30 mol %). The vesicle preparation

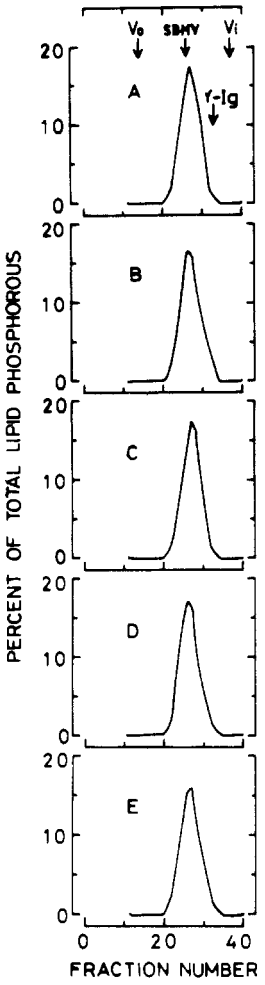

FIGURE 2: Elution patterns of vesicles from Bio-Gel A-50m column. For details, see Materials and Methods. (A) SM-**2a**; (B) SM-**2b**; (C) SM-**2c**; (D) SM-**2d**; (E) SM-**2e**.

was divided into two equal portions. After addition of methanol/diethyl ether (2:98 v/v) to one of the portions, both the portions were incubated with phospholipase A_2 essentially under the conditions as described above. Aliquots removed at different time intervals were treated with equal volumes of 100 mM EDTA. Lipids were extracted. The extracts, after being concentrated under N_2, were applied to silica gel plates that were developed in chloroform/methanol/water (65:25:4 v/v). The spots were visualized by iodine staining. Total phosphorus in each spot was determined as described above.

Results and Discussion

The structural changes that were introduced in the polar head group of **2a** to obtain **2b–e** were based on similar considerations as discussed earlier for phosphatidylethanolamines **1a–e** (Kumar & Gupta, 1983). SUV from binary mixtures of SM and PC were formed by sonication (pH 7.4) and fractionated by centrifugation. Only the vesicles containing about 30 mol % of one of the phosphatidylcholines **2a–e** were considered appropriate for the study. This concentration of PC selected as an increased mole fraction of this lipid (50 mol %) in the vesicles resulted in markedly enhanced release rates of the entrapped solute during phospholipase A_2 treatments. On the other hand, reducing the vesicle PC levels to 15 mol % led to difficulties in accurately estimating the amounts of lyso-PC due to its partial mixing with the highly diffused band of SM on the TLC plates.

The vesicle size was determined by molecular sieve chromatography on Bio-Gel A-50m. Figure 2 shows that in spite

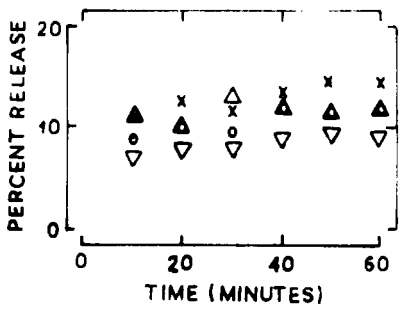

FIGURE 3: Efflux of the entrapped 6-CF from SM-**2a** vesicles during phospholipase A_2 treatments. Fully quenched concentrations of 6-CF (Weinstein et al., 1977) were entrapped in the vesicles. Free and trapped dye were separated by gel-permeation chromatography over a Sephadex G-50 column (1.4 × 40 cm). The vesicles were incubated separately with buffer (○), 10 mM Ca^{2+} (△), phospholipase A_2 and 10 mM Ca^{2+} (×), and phospholipase A_2 and 5 mM EDTA (▽) at 25–30 °C. pH of the incubation mixture was about 8.5. Measured aliquots from these incubation mixtures were withdrawn at specified periods of time, and the amounts of total and free dye were determined by measuring fluorescence in the presence as well as in the absence of Triton X-100 (1% final concentration). Percent of 6-CF release was calculated from $100 \times dye_f/dye_t$, where dye_f and dye_t denote free and total dye, respectively. Degradation of PC in the vesicles that were treated with phospholipase A_2 in the presence of Ca^{2+} was established by TLC analysis.

of the differences in the PC head-group structure the elution profiles of SM-modified PC vesicles (B–E) were almost identical with those obtained for SM-**2a** vesicles (A), indicating that the structural alterations introduced in the head group of **2a** do not significantly affect the vesicle size. The Stokes radius of these vesicles was calculated as described by Ackers (1967), with SBMV and γ-Ig as the column markers. These vesicles were found to have a Stokes radius of about 150 Å. This is quite consistent with the internal volume of the vesicles (0.6–0.7 μL/μmol of lipid P) determined by [^{14}C]glucose entrapment.

Transbilayer PC distributions in SM–PC vesicles were ascertained by analyzing the accessibility of this phospholipid to phospholipase A_2 in intact vesicles (Sundler et al., 1978; Kupferberg et al., 1981; Kumar & Gupta, 1984). That the structural integrity of the vesicles is fully retained during or after their treatments with the enzyme was confirmed by examining the efflux of the entrapped solutes and also the elution patterns of the hydrolyzed vesicles from the Bio-Gel column. Figure 3 shows that the leakage rates of the entrapped 6-CF were not significantly enhanced during hydrolysis of SM-**2a** vesicles by phospholipase A_2. Also, the elution profiles of vesicles from Bio-Gel A-50m remained unaffected after their treatments with the enzyme (Figure 4). It is therefore inferred from these experiments that the vesicles' hydrolysis by phospholipase A_2 neither causes vesicles lysis nor vesicle fusion.

Previous studies have shown that phospholipase A_2 in intact vesicles hydrolyzes only the glycerophospholipids that are present in the outer monolayer (Sundler et al., 1978; Kupferberg et al., 1981; Kumar & Gupta, 1984). This was established in the present study by analyzing the kinetics of PC hydrolysis in intact as well as in lysed vesicles. In the intact SM-**2a** vesicles, phospholipase A_2 hydrolyzed about 50% **2a** in 2 h. These amounts did not further increase with time up to 6 h (Figure 5). In identical conditions, this enzyme in the lysed vesicles readily cleaved about 93% **2a** in 1 h. This demonstrates that phospholipase A_2 in intact SM–PC vesicles degrades only that fraction of PC that is localized in the outer surface of the SUV bilayer and that the internal pool of **2a** does not rapidly exchange with the external **2a** in these vesicles. The latter finding is consistent with the earlier studies that

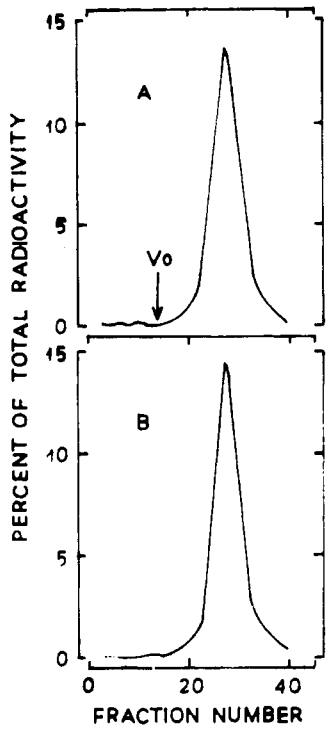

FIGURE 4: Elution profile of phospholipase A_2 hydrolyzed SM–**2a** vesicles, containing traces of [^{14}C]SM, from a Bio-Gel A-50m column (1 × 50 cm): (A) vesicles before enzyme treatments; (B) vesicles after treating them with phospholipase A_2 for 3 h. V_0 is the void volume.

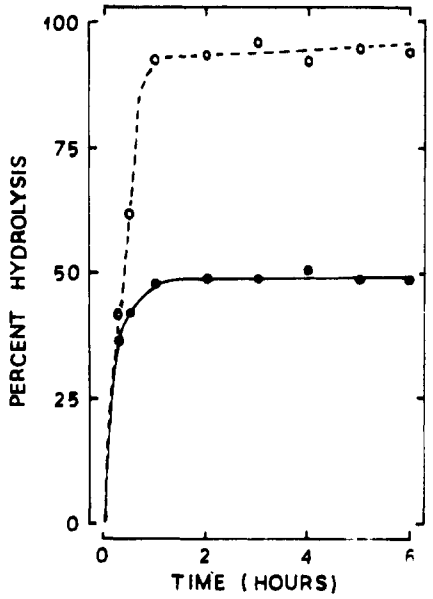

FIGURE 5: Kinetics of hydrolysis of **2a** in SM–**2a** vesicles by phospholipase A_2: (●) intact vesicles; (○) vesicles lysed with 2% methanol in diethyl ether.

showed that transbilayer exchange (flip-flop) of phospholipids in model membrane systems is a very slow process (Op den Kamp, 1979).

These experiments besides demonstrating that phospholipase A_2 in intact SM–PC vesicles hydrolyzes only the external PC also establish that treatments of the vesicles with the enzyme do not induce alterations in the vesicles structural integrity. This enzyme was therefore used to probe the transbilayer PC distributions in the various vesicle preparations. Data given in Table I indicate that phospholipase A_2 in intact SM–**2a** vesicles hydrolyzed only about 45% of the total vesicle **2a**. But

Table I: Vesicle Phospholipid Degradation by Phospholipase A_2

vesicles	vesicle lipid composition[a] PC (%)	SM (%)	PC degradation (%)[a]
SM–POPC	27.7 ± 0.5	72.3 ± 0.5	44.6 ± 1.3
SM–**2a**	28.3 ± 0.7	71.7 ± 0.7	46.9 ± 1.7
SM–**2b**	30.5 ± 1.8	69.5 ± 1.8	78.7 ± 2.0
SM–**2c**	28.6 ± 0.6	71.4 ± 0.6	69.5 ± 0.5
SM–**2d**	28.4 ± 0.9	71.6 ± 0.9	79.8 ± 1.3
SM–**2e**	30.5 ± 0.6	69.5 ± 0.6	82.1 ± 0.8

[a] Values are mean of six determinations ± SD.

Table II: Vesicle Phospholipid Degradation by Phospholipase A_2 in the Presence of Bovine Serum Albumin

vesicles	vesicle lipid composition[a] PC (%)	SM (%)	PC degradation (%)[a]
SM–POPC	30.2 ± 0.7	69.8 ± 0.7	50.2 ± 0.5
SM–**2a**	30.6 ± 0.4	69.4 ± 0.4	50.7 ± 0.6
SM–**2b**	29.7 ± 0.2	70.3 ± 0.2	78.2 ± 0.3
SM–**2c**	29.3 ± 0.5	70.7 ± 0.3	69.7 ± 0.8
SM–**2d**	29.1 ± 0.6	70.9 ± 0.6	80.8 ± 1.5
SM–**2e**	28.6 ± 0.3	71.4 ± 0.3	81.7 ± 0.4

[a] Values are mean of four to six determinations ± SD.

Table III: Vesicle Phospholipid Degradation by Phospholipase A_2 in the Presence of 2% Methanol in Diethyl Ether

vesicles	vesicle lipid composition[a] PC (%)	SM (%)	PC degradation (%)[a]
SM–POPC	29.8 ± 0.9	70.2 ± 0.9	94.5 ± 2.0
SM–**2a**	30.3 ± 0.5	69.7 ± 0.5	93.3 ± 2.2
SM–**2b**	31.3 ± 0.6	68.7 ± 0.6	93.8 ± 1.9
SM–**2c**	30.4 ± 1.1	69.6 ± 1.1	94.6 ± 1.3
SM–**2d**	30.0 ± 0.8	70.0 ± 0.8	93.9 ± 2.7
SM–**2e**	28.3 ± 0.3	71.7 ± 0.3	96.0 ± 0.9

[a] Values are mean of four to six determinations ± SD.

in identical conditions, this enzyme readily degraded about 79% **2b**, 70% **2c**, 80% **2d**, and 82% **2e** in intact vesicles. The lower extents of PC hydrolyses in SM–**2a** vesicles were not due to inhibition of the enzyme activity by lyso-**2a** formed during the phospholipase A_2 treatments (Kupferberg et al., 1981), as the amounts of hydrolyzed **2a** did not significantly increase after including 9.2×10^{-5} M bovine serum albumin in the incubation mixture (Table II). Furthermore, the higher amounts of the hydrolyzed **2b–e**, as compared to that of the hydrolyzed **2a**, did not arise from vesicles lysis during the enzyme reactions since incubations of the lysed vesicles with phospholipase A_2, in identical conditions, resulted in almost complete (over 94%) hydrolyses of **2b–e** (Table III). Neither can the above differences between the amounts of hydrolyzed **2a** and **2b–e** be attributed to differences in the size of vesicles because the various SUV preparations were shown to have similar Stokes radii and internal volumes. From these experimental facts, we conclude that the observed differences between the accessibility of **2a** and its analogues to phospholipase A_2 in intact vesicles are primarily due to changes in the PC distributions across the vesicles bilayer.

In small unilamellar egg PC vesicles (Stokes radius 105 Å), the ratio of external lipid to the internal lipid is about 2 (Huang & Mason, 1978). This ratio in the SM–PC vesicles on the one hand should decrease due to increase in the Stokes radius, but on the other, it is expected to increase as the thickness of SM bilayers is about 25% greater than that of the PC bilayers (Schmidt et al., 1977). The external PC to internal PC ratios that are appreciably lesser or greater than 2 would, therefore,

Table IV: Distributions of Phosphatidylcholines[a] in Small Unilamellar SM–PC Vesicles

vesicles	$[PC]_e$ (mol %)	$[PC]_i$ (mol %)	$[PC]_e/[PC]_i$
SM–POPC	15.16	15.04	1.01
SM–**2a**	15.51	15.09	1.03
SM–**2b**	23.22	6.48	3.58
SM–**2c**	20.42	8.88	2.30
SM–**2d**	23.51	5.59	4.21
SM–**2e**	23.37	5.23	4.47

[a] The mol fractions of external PC ($[PC]_e$) and internal PC ($[PC]_i$) were calculated from the values of total vesicle PC and hydrolyzed PC as shown in Table II.

signify the asymmetric distributions of this lipid across the SM–PC vesicles bilayer. Table IV shows that this ratio in SM–**2a** vesicles is only about 1, suggesting that in these vesicles **2a** is preferentially distributed in the inner monolayer. This distribution does not seem to be influenced by the nature of acyl chains in PC, as replacement of **2a** in the vesicles by POPC had no effect on the external PC to internal PC ratio (Table IV). These results are in agreement with the earlier studies that showed that PC in highly curved SM–PC vesicles invariably prefers the inner monolayer (Berden et al., 1974, 1975; Castellino, 1978). However, this preference of PC in these vesicles is dramatically altered upon introducing C_2H_5, C_6H_5, and $C_6H_5CH_2$ substituents at the carbon atom adjacent to the quaternary ammonium group. The so-derived PC analogue **2c** seemed to distribute symmetrically across the SM–**2c** vesicles bilayer whereas **2d** and **2e** were predominantly localized in the outer monolayer. Similarly, the PC derivative **2b**, wherein the phosphate and quaternary ammonium groups were separated by three CH_2 residues, also preferred the exterior side in SM–**2b** vesicles.

Substitutions by C_2H_5, C_6H_5, and $C_6H_5CH_2$ groups are not likely to alter the charge properties of the **2a** head group but could affect the head-group volume, the phospholipid conformation, and the charge–charge repulsion between head groups in the bilayer. Alterations in the charge–charge repulsion must alter the head-group separation in the bilayer and should, therefore, lead to differences between the permeability properties of the SM–**2a** and SM–**2c** (or **2d**,**e**) vesicles and also between the melting phase transition temperatures ("T_m") of **2a** and **2c–e**. But replacement of **2a** by **2c–e** in SM–PC bilayers was not found to affect the efflux rates by [^{14}C]glucose from the vesicles. Also, the "T_m" values for **2c–e** (40–42 °C), as determined by differential scanning calorimetry, were quite similar to that observed for **2a** (41 °C). From these findings, it may, therefore, be concluded that the observed differences between the transbilayer distributions of **2a** and its analogues primarily arise from the differences in their head-group volumes rather than in their conformations or the charge–charge repulsion between the head groups. As the relative distributions of PC in SM–PC vesicles were found to depend on the chemical structure of the PC head group, it may be further concluded that the phospholipid distributions across the vesicles bilayer are largely determined by the differences between the head-group volumes of SM and the phosphatidylcholines **2a–e**. This is consistent with the earlier studies (Nordlund et al., 1981; Kumar & Gupta, 1983) that have suggested that the transbilayer phospholipid distributions in highly curved vesicles are controlled mainly by the phospholipid head-group volume rather than by the head-group charge (Berden et al., 1975; Massari et al., 1978; Lentz et al., 1980).

Hydration characteristics, surface area per lipid molecule, and head-group conformation for SM are similar to those for the structurally related PC (Shipley et al., 1974; Khare & Worthington, 1978). However, the intramolecular hydrogen bonding between the phosphate and the hydroxyl or amide group in SM (Barenholz & Thompson, 1980) may, apparently, lead to a larger effective volume of its polar head group, as compared to that of **2a**. Since the surface area per lipid head group available in the outer monolayer is greater than that in the inner monolayer (Huang & Mason, 1978), the preference of SM for the outer surface in highly curved SM–**2a** vesicles is quite understandable. This preference probably disappears in SM–**2c** vesicles and is reversed in the vesicles comprised of SM and **2b**, **2d**, or **2e**. In these cases, it may be presumed that the effective head-group volume of SM is either similar to or smaller than the PC head group.

In summary, this study clearly demonstrates that transbilayer distributions of **2a** in SUV containing SM may be regulated by affecting appropriate changes in the **2a** head group. As such, **2a** distributes preferentially in the inner leaflet of the vesicles bilayer, but this preference is reversed upon introducing C_6H_5 and $C_6H_5CH_2$ substituents in the **2a** head group as well as on increasing the **2a** head group length. In addition, nearly symmetric distributions of **2a** across the vesicles bilayer are observed on introducing one C_2H_5 group in the choline moiety. These differences in phospholipid distributions primarily arise from the differences between the effective head-group volumes of **2a–e** and SM.

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Comparison of Cytosolic and Nuclear Poly(A) Polymerases from Rat Liver and a Hepatoma: Structural and Immunological Properties and Response to NI-Type Protein Kinases†

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ABSTRACT: Poly(A) polymerases were purified from the cytosol fraction of rat liver and Morris hepatoma 3924A and compared to previously purified nuclear poly(A) polymerases. Chromatographic fractionation of the hepatoma cytosol on a DEAE-Sephadex column yielded approximately 5 times as much poly(A) polymerase as was obtained from fractionation of the liver cytosol. Hepatoma cytosol contained a single poly(A) polymerase species [48 kilodaltons (kDa)] which was indistinguishable from the hepatoma nuclear enzyme (48 kDa) on the basis of CNBr cleavage maps. Liver cytosol contained two poly(A) polymerase species (40 and 48 kDa). The CNBr cleavage patterns of these two enzymes were distinct from each other. However, the cleavage pattern of the 40-kDa enzyme was similar to that of the major liver nuclear poly(A) polymerase (36 kDa), and approximately three-fourths of the peptide fragments derived from the 48-kDa species were identical with those from the hepatoma enzymes (48 kDa). NI-type protein kinases from liver or hepatoma stimulated hepatoma nuclear and cytosolic poly(A) polymerases 4–6-fold. In contrast, the liver cytosolic 40- and 48-kDa poly(A) polymerases were stimulated only slightly or inhibited by similar units of the protein kinases. Antibodies produced in rabbits against purified hepatoma nuclear poly(A) polymerase reacted equally well with hepatoma nuclear and cytosolic enzyme but only 80% as well with the liver cytosolic 48-kDa poly(A) polymerase and not at all with liver cytosolic 40-kDa or nuclear 36-kDa enzymes. Anti-poly(A) polymerase antibodies present in the serum of a hepatoma-bearing rat reacted with hepatoma nuclear and cytosolic poly(A) polymerases to the same extent but only 40% as well with the liver cytosolic 48-kDa enzyme. From this value and the relatively low quantity of liver 48-kDa poly(A) polymerase, it is calculated that per unit weight of tissue, adult liver contains only 3% of the tumor poly(A) polymerase determinants which are immunogenic in an allogenic host. On the basis of the CNBr cleavage maps, immunological characteristics, and response to NI-type protein kinases, it is concluded that (a) hepatoma cytosol contains a single poly(A) polymerase that is identical with the hepatoma nuclear enzyme and (b) liver cytosol contains two distinct poly(A) polymerases, one (48 kDa) similar to but not identical with the hepatoma enzyme (48 kDa) and the other (40 kDa) possibly related to the major liver nuclear enzyme (36 kDa).

Several years ago, nuclear poly(A) polymerase from the rat tumor Morris hepatoma 3924A was shown to differ from the corresponding liver enzyme by a number of criteria (Rose & Jacob, 1976, 1979; Rose et al., 1978). Recent studies in our laboratory have demonstrated that nuclear poly(A) polymerases from the hepatoma (M_r 48 000) and liver (M_r 36 000–38 000) are structurally and immunologically distinct (Stetler & Jacob, 1984). These studies also demonstrated the existence of a minor (1% of total) liver nuclear poly(A) polymerase that was identical with the hepatoma nuclear enzyme with respect to immunological characteristics, molecular weight, and CNBr cleavage map. Because the same 48-kilodalton (kDa) enzyme was the major species isolated from fetal rat liver nuclei (Stetler & Jacob, 1985), this enzyme could be the product of an oncofetal gene which is expressed only to a limited extent in normal adult liver. However, poly(A) polymerase has also been identified in other cellular fractions including mitochondria (Jacob et al., 1972; Rose et al., 1975; Aujame & Freeman, 1976), microsomes (Wilke & Smellie, 1968; Rose

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