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TRANSBILAYER DISTRIBUTIONS OF RED CELL MEMBRANE PHOSPHOLIPIDS IN UNILAMELLAR VESICLES *

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The phospholipid organization in unilamellar vesicles comprised of various purified phospholipid components of monkey erythrocyte membrane was ascertained using phospholipase A_2 and trinitrobenzenesulfonic acid as external membrane probes. The vesicles were formed by sonication or detergent dialysis and fractionated by centrifugation or gel permeation chromatography. Experiments were done to confirm that the phospholipase A_2 treatments did not cause lysis or induce fusion of the vesicles. This enzyme hydrolysed only the glycerophospholipids in the outer surface of the vesicles. The amounts of the external phospholipids determined by this enzymatic method were verified using the chemical probe, trinitrobenzenesulfonic acid. The choline-containing phospholipids and phosphatidylethanolamine localized randomly in the two surfaces of sonicated vesicles (outer diameter, about 30 nm), whereas phosphatidylserine preferentially distributed in the inner monolayer. This phosphatidylserine asymmetry virtually disappeared in detergent dialysed vesicles (outer diameter, about 45 nm). Furthermore, inclusion of cholesterol in both the types of vesicles resulted in more random glycerophospholipid distributions across the plane of vesicles bilayer, presumably due to the cholesterol-induced increases in the size of vesicles. These results demonstrate that the transbilayer distribution of erythrocyte membrane phospholipids in unilamellar vesicles are controlled mainly by the surface curvature rather than by interlipid interactions, and therefore suggest that phospholipid-phospholipid and phospholipid-cholesterol interactions should not play any significant role in determining the membrane phospholipid asymmetry in red cells. It is proposed that this asymmetry primarily originates from differential bindings of phospholipids with membrane proteins in the two leaflets of the membrane bilayer.

Introduction

Various membrane components are asymmetrically localized in two halves of membrane bilayer [1,2]. Generally, the proteins are distributed with an absolute asymmetry while the various classes of phospholipids are present in both monolayers, albeit in unequal amounts. In erythrocyte membrane, phosphatidylcholine (PC) and sphingomyelin (SM) are localized mainly in the outer monolayer whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) are present almost exclusively in the inner monolayer [3].

Several investigators have attempted to understand the intermolecular interactions that might induce asymmetric distribution of phospholipids across the plane of red cell membrane bilayer by studying binary mixtures of phospholipids in unilamellar vesicles [4–9]. It has been shown that PE

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; phosphatidylserine; PI, phosphatidylinositol; TNBS, trinitrobenzenesulfonic acid.

in small unilamellar vesicles consisting of PC and PE (> 10 mol%) distributes preferentially in the inner monolayer [4,5] while SM in PC/SM vesicles prefers the outer monolayer [6]. Furthermore, PS in small vesicles prefers the inner monolayer [6], at least at low PS concentrations [7].

To evaluate the significance of the above findings in terms of the factors that induce membrane phospholipid asymmetry in red cells, we have studied the transbilayer distributions of monkey erythrocyte membrane phospholipids in unilamellar vesicles. The vesicles were prepared in the absence as well as in the presence of 40 weight % cholesterol from the phospholipid mixture consisting of PC, SM, PE and PS in a ratio similar to that observed in erythrocytes, and were fractionated by centrifugation or gel permeation chromatography. Phospholipase A_2 and trinitrobenzenesulfonic acid (TNBS) were used as external membrane probes to study the phospholipid organization in vesicles bilayer. The experiments were carefully controlled to assure that the reagents did not lyse or permeate the vesicles. The results of these studies show that the transbilayer distributions of erythrocyte membrane phospholipids in unilamellar vesicles are determined mainly by the packing constraints imposed by the surface curvature rather than by some specific interlipid interactions.

Materials and Methods

6-Carboxyfluorescein and TNBS were from Eastman Kodak Company and Sigma Chemical Company, respectively. Phospholipase A_2 was purified from *Naja naja* snake venom (Haeffkine Institute, Bombay) by the method of Blecher [10], to a protein concentration of approx. 1 mg/ml. [^{3}H]Cholesterol and [^{3}H]inulin were purchased from the Radiochemical Center, Amersham, U.K., and New England Nuclear, respectively. Egg [*methyl*-^{14}C]PC (30 μCi/μmol) was prepared as described by Gupta and Bali [11]. Sephadex LH-20 (25–100 μm beads) and Sephadex G-50 (20–80 μm beads) were obtained from Pharmacia Fine Chemicals. Bio-Gel A-50m was from Bio-Rad Laboratories. Pre-coated silica gel 60F-254 TLC plates (20 × 20cm, 0.2 mm thickness) were bought from E. Merck. Silica gel (60–120 mesh, activity 2–3) was purchased from Sisco Research Laboratories, Bombay, India.

The assay of radioactive isotopes was carried out in a Packard Tri-Carb 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.

Isolation and purification of phospholipids from monkey erythrocyte membrane

Lipids from erythrocytes of freshly drawn rhesus monkey blood were isolated by the procedure of Rose and Oklander [12]. Individual phospholipid components from the mixture were separated by chromatography over silica gel (Table I) and Sephadex LH-20. The Sephadex LH-20 chromatography was performed as described earlier [13]. Although chromatography over silica gel column separated most of the phospholipid components, further chromatography of each phospholipid component on Sephadex LH-20 was necessary to separate phosphatidylinositol (PI) from traces of PE, PS from the presumed glycolipid component, and traces of colored impurities from PE. Pure PC, PE, PS and PI were obtained by this procedure. However, SM could not be made free from PC by this method. Therefore, the impure SM was first treated in diethyl ether with methanolic solution of tetra-*n*-butylammonium hydroxide and then, after removal of the solvents, the mixture was chromatographed on Sephadex LH-20. This led to the isolation of pure SM in reasonable amounts.

Purity of phospholipids was examined by TLC on silica gel G-60 plates using chloroform/methanol/water (65 : 25 : 4, v/v) as the developing solvent system. The spots were identified after staining the plate with iodine vapor followed by molybdenum-blue spray [14]. All the samples exhibited single spots. Each phospholipid component was characterized by its co-chromatography with the analytical samples, prepared as reported earlier [15]. These were stored as solutions in chloroform/methanol (1 : 1, v/v) at −20°C under N_2.

Separation of phospholipids by TLC

Separation of various phospholipids was done by two-dimensional TLC on E. Merck silica gel TLC plates as described by Pollet et al. [16]. Spots

TABLE I

SEPARATION OF MONKEY ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS ON SILICA GEL COLUMN

The column (0.6 × 40 cm, 5.0 g silica gel) was washed with 100 ml of chloroform (free of ethanol) prior to applying the sample. About 250 mg of the crude mixture was applied in a total volume of about 2.0 ml of chloroform. The flow rate was 1.5–2.0 ml/min. 5 ml fractions were collected. The fractions were analysed by TLC.

Solvent No.	Solvent composition ratio (v/v)		Volume of eluate (ml)	Lipid eluted
	Chloroform	Methanol		
1	100	0	100	Neutral lipids
2	95	5	100	Colored impurities
3	90	10	50	PE
4	85	15	50	PI + traces of PE
5	80	20	50	Traces of some unknown lipid
6	75	25	50	PS
7	70	30	60	Some glycolipid + traces of PS
8	65	35	50	PC
9	60	40	350	SM + traces of PC
10	50	50	100	No other phospholipid

for different phospholipids were identified after staining the plate with iodine vapor followed by ninhydrin spray. These were removed and eluted with a mixture of methanol and chloroform (1 : 1, v/v) for several times. Total phosphorus present in each spot was determined as described by Ames and Dubin [17]. The recoveries of various phospholipids from silica gel were over 95%.

Preparation of unilamellar vesicles by sonication

A solution of PC, PE, SM and PS in the absence as well as in the presence of 40 weight % cholesterol 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 solvents were removed by leaving the tube in vacuo for 3–4 h. The lipid mixture was dispersed in Tris-buffered or phosphate-buffered saline (10 mM in 0.9% NaCl, pH 7.4) so as to achieve a concentration of about 2.5–5.0 μmol lipid P/ml buffer. It was vortexed for 5–10 min at room temperature. The lipid dispersion so obtained was carefully transferred to a cuvette and sonicated at 0°C (or $\leqslant$ 20°C) under N_2 using a probe-type sonicator (MSE 150 W, 20 KHz) to give an optically clear suspension (30–45 min). 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 vesicles found in the top two-thirds of the supernatant were used.

Preparation of unilamellar vesicles by detergent dialysis

The dried phospholipid mixture was dispersed in Tris-buffered or phosphate-buffered saline (10 mM in 0.9% NaCl, pH 7.4) containing 2% cholic acid and 0.02% NaN_3 (w/v) so as to achieve the cholate to phospholipid molar ratio of about two. It was vortexed and then dialysed against 10 mM Tris or phosphate (pH 7.4) containing 0.9% NaCl and 0.02% NaN_3 (4 × 500 ml) at 20°C. After dialysis, the vesicles were fractionated by centrifugation as described above. The amount of cholate that could not be removed by dialysis was determined by including trace amounts of [^{3}H]cholic acid in the lipid/detergent mixture. The molar ratio of phospholipid to cholate in vesicles was approx. 230.

Determination of vesicle size distribution

Homogeneity of the vesicle preparations was analysed by column chromatography on Bio-Gel A-50m at 20°C. A downward flowing column (1 × 50 cm) of Bio-Gel A-50m maintained at constant hydrostatic pressure was equilibrated with 10 mM Tris-HCl buffer (pH 7.4). A measured aliquot

(0.5 ml) of the vesicle preparation was applied to the column and eluted with the same buffer at 8 ml/h. Fractions were analysed by measuring total phosphorus.

Determination of lipid compositions of vesicle

Lipids from vesicles were extracted by Bligh-Dyer extraction procedure [18]. After removal of solvents from the extracts, the dried lipid mixture was dissolved (approx. 4 μg lipid P/μl) in chloroform/methanol (1 : 1, v/v) mixture. This solution was applied to E. Merck silica gel TLC plates (25–50 μl/plate) and different phospholipid species were separated as described above. The percentage incorporation of cholesterol in the bilayers was ascertained by including about 5 μCi of [^{3}H]cholesterol in the lipid mixture and then determining the ratio of lipid P to ^{3}H in vesicles. At least 90% of the added amount of cholesterol was found to be incorporated in phospholipid bilayers.

Preparation of vesicles containing trapped [^{3}H]inulin (or carboxyfluorescein)

Unilamellar vesicles containing trapped [^{3}H]inulin or carboxyfluorescein were prepared by dispersing the lipid mixture in buffer containing [^{3}H]inulin (approx. 5 μCi/ml) or carboxyfluorescein (0.2 M). Free and trapped [^{3}H]inulin (or carboxyfluorescein) were separated by gel filtration on Bio-Gel A-50m (1 × 50 cm column). The fractionated vesicles were concentrated to about 1 ml in an Amicon Centriflo CF-25 cone.

Hydrolysis of vesicle phospholipids by phospholipase A_2

Vesicles (2–4 μmol lipid P) were incubated with 20 μg of phospholipase A_2 in Tris-buffered saline (10 mM in 0.9% NaCl containing 10 mM $CaCl_2 \cdot 2H_2O$, pH 8.5) in a total volume of 2.0 ml for 2–3 h at 25–30°C. The reactions were terminated by adding 50 mM EDTA (1.0 ml). The lipids were extracted and the intact and hydrolyzed phospholipids were separated by two-dimensional TLC.

Kinetics of hydrolysis of vesicle PC by phospholipase A_2

For studying the kinetics of hydrolysis of vesicle PC, unilamellar vesicles were prepared after including trace amounts of egg [^{14}C]PC (about 1 μCi) in the lipid mixture. The vesicle preparation was divided in two equal portions. After adding Triton X-100 (0.5%) or bovine serum albumin ($9.2 \cdot 10^{-5}$M) 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 50 mM EDTA. Lipids were extracted. The extracts, after concentrating under N_2, were applied to silica gel G-60 TLC plates that were developed in chloroform/methanol/water (65 : 25 : 4, v/v). PC and lysoPC were visualized by autoradiography. The spots corresponding to PC and lysoPC were removed and assayed for radioactivity.

Labeling of vesicles with TNBS

Unilamellar vesicles were prepared from the lipid mixture in phosphate-buffered saline (10 mM in 0.9% NaCl, pH 7.4) by sonication or detergent dialysis as described above. The pH of the vesicle preparation was raised to 8.2. To a part of this preparation (2–4 μmol lipid P) was added a solution of TNBS in aqueous $NaHCO_3$ (pH 8.2) so as to give a final concentration of 20 mM of the reagent in a total volume of 2.0 ml. The mixture was incubated at 20 ± 2°C for 40–60 min. After this period, the reactions were terminated by adding 1 M HCl till pH of the reaction mixture was about 4.0. The lipids were extracted. The labeled and unlabeled phospholipids after separation by two-dimensional TLC were estimated as described by Gupta and Misra [19].

Kinetics of TNBS labeling of vesicles

Kinetics of TNBS labeling of aminophospholipids in vesicles was studied by modifying the published procedure [8]. To an aliquot (40 μml) of vesicle preparation, containing not more than 0.25 μmol of aminophospholipids, were added 10 mM phosphate buffer (pH 8.5; 560 μl) and 0.8 M $NaHCO_3$ (200 μl). After vortexing the mixture, a 20 μl aliquot of 1.5% TNBS solution in 0.8 M $NaHCO_3$ was added and the mixture was incubated at 20 ± 2°C in dark for different time intervals. The reaction was terminated by adding 1.2% Triton X-100 (400 μl) in 1.5 M HCl. Absorbance at 410 nm was read within 1 h after terminating the reaction. The total aminophospho-

lipid content in vesicles was determined as follows: an aliquot (40 μl) of the same vesicle preparation as above 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 and the mixture was incubated in dark at $20 \pm 2°C$ for the same time intervals as above. After incubation, 0.4% Triton X-100 in 1.5 M HCl (400 μl) was added. The 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 various phospholipid components of monkey erythrocyte membrane were purified as described in Materials and Methods. The pure major components (PC, PE, SM and PS) were mixed so as to approximate their ratio in the erythrocyte membrane. Unilamellar vesicles from the phospholipid mixture were prepared in the absence as well as in the presence of 40 weight % cholesterol by sonication or detergent dialysis, and fractionated by centrifugation or gel filtration.

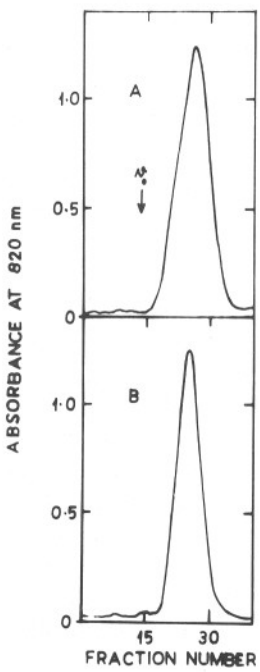

Fig. 1. Elution patterns of vesicles from Bio-Gel A-50m column. (A) Sonicated vesicles. (B) Detergent dialysed vesicles. Multilamellar vesicles were used as void volume (V_0) marker.

Homogeneity of the vesicle preparations was routinely analysed by gel permeation chromatography on Bio-Gel A-50m. These vesicles invariably eluted with the included volume of the Bio-Gel column (Fig. 1). As may be seen in Fig. 1, the detergent dialysed vesicles eluted faster than the sonicated vesicles from the column. Apart from this difference between the two types of vesicles, the peak width at half height was smaller for the detergent dialysed vesicles (Fig. 1). These observations clearly suggest that the vesicles formed by detergent dialysis are lesser heterogeneous and larger in size than that formed by sonication. This was further confirmed by measuring the outer diameters of both the types of vesicles, using negative staining electron microscopy. The average outer diameters of the sonicated and the detergent dialysed vesicles were about 30 nm (size range 25–50 nm) and 45 nm (size range 35–50 nm), respectively. Also, these average sizes of vesicles were consistent with the amounts of trapped solutes. Inclusion of 40 weight% cholesterol in the phospholipid bilayers did not change considerably the size range of both the types of vesicles, as evidenced by their elution patterns from the Bio-Gel column, but appeared to induce an appreciable increase in the average size of vesicles. The relative ratio of the various phospholipid components in the vesicles bilayer was also determined and found to be similar to that observed in the erythrocyte membrane (Table II).

The transbilayer distributions of glycerophospholipids in these vesicles were ascertained using phospholipase A_2 and TNBS as the external membrane probes [4,20]. Experiments were done to confirm that the structural integrity of the vesicles is retained during or after their hydrolysis with phospholipase A_2. Vesicles containing [^{3}H]inulin or carboxyfluorescein as the trapped solute were used in these experiments. The vesicle stability was ascertained by (a) measuring the efflux of the entrapped solutes, and (b) gel filtration of the hydrolysed vesicles. Leakage of the trapped solutes remained unaffected by phospholipase A_2 treatments of the vesicles (Figs. 2 and 3). Also, the elution profiles of the hydrolysed vesicles from

TABLE II

MEMBRANE PHOSPHOLIPID COMPOSITIONS

Each value is a mean of 10–15 determinations ± S.D.. The values shown for PC include the amounts of the corresponding lyso derivative also. The amounts of this lysolipid in the lipid extracts of red cells and vesicles were 0–2% and 1–3%, respectively. No other lysolipid was present in these extracts.

Membrane	% of total phospholipids				
	PC	PE	PS	SM	PI
Monkey red cell	38.8 ± 1.0	32.7 ± 0.8	11.3 ± 0.7	14.6 ± 1.0	2.6 ± 0.4
Vesicles	39.5 ± 2.1	29.3 ± 2.4	10.4 ± 2.6	20.6 ± 2.0	0

Bio-Gel A-50m were similar to that observed for intact vesicles (Fig. 3, A and B). These findings completely exclude the possibility of vesicle lysis during the enzyme hydrolysis. Moreover, the observed similar elution patterns of the vesicles before and after the enzyme (or Ca^{2+}) treatment

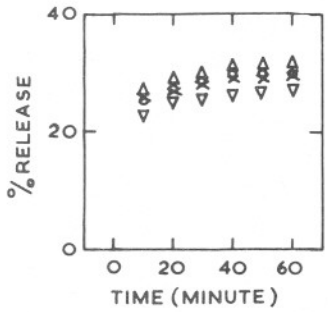

Fig. 2. Efflux of the entrapped 6-carboxyfluorescein from vesicles during phospholipase A_2 treatments. Fully quenched concentrations of 6-carboxyfluorescein [38] were entrapped in vesicles. Free and the trapped dye were separated by gel permeation chromatography (see Materials and Methods). After this chromatography, 20–25% of the total dye eluted with vesicles was characterized as free 6-carboxyfluorescein. No attempts were, however, made to remove this amount of free dye from the vesicles. These vesicles were incubated separately with buffer (○), 10 mM Ca^{2+} (△), phospholipase A_2 (5 μg protein/μmol lipid P) 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 determined by measuring fluorescence in the presence as well as in the absence of Triton X-100 (1% final concentration) on an Aminco SPF-500 fluorimeter using excitation and emission wavelengths of 490 and 520 nm, respectively. Percent 6-carboxyfluorescein release was calculated from $100 \times Dye_f/Dye_t$, where Dye_f and Dye_t denote free and total dye, respectively. Degradation of phospholipids in the vesicles that were treated with phospholipase A_2 in the presence of Ca^{2+} was established by TLC analysis.

(Fig. 3, A–C) also rule out the possibility of the vesicle fusion in these experiments. This was further established by our observation that the vesicle size, as measured by negative staining electron microscopy, was not much affected after treating the vesicles with phospholipase A_2.

Earlier studies have revealed that phospholipase A_2 hydrolyses only the phospholipids in the outer leaflet of the vesicle bilayer [20,21]. In order to verify these findings in the present case, the kinetics of the enzyme hydrolysis of the vesicle PC was examined as described in Materials and Methods. The results shown in Fig. 4 indicate that during the initial phase of the enzyme reaction the hydrolysis in the intact vesicles proceeded much faster than that in the vesicles lysed with Triton X-100. In the first 30 min approx. 70% of the total vesicle PC was hydrolysed. After this period, there were no further increases in the amounts of the hydrolysed PC upto 6 h. Moreover, these amounts were not affected even by including $9.2 \cdot 10^{-5}$ M bovine serum albumin in the incubation mixtures, excluding the possibility of inhibition of the enzyme activity by the reaction products [21]. However, addition of methanol/ether (2 : 98, v/v) to these reactions led to the complete hydrolysis of all the glycerophospholipids.

These experiments strongly indicate that phospholipase A_2 hydrolyses only the phospholipids in the exterior side of the unilamellar vesicles, without affecting the vesicle structural integrity. Therefore, this enzyme was used to study the transbilayer phospholipid distributions in various vesicle preparations. The results given in Table III indicate that the enzyme invariably degraded appreciably higher amounts of PC than that of PE and PS in vesicles. These differences in the amounts were not

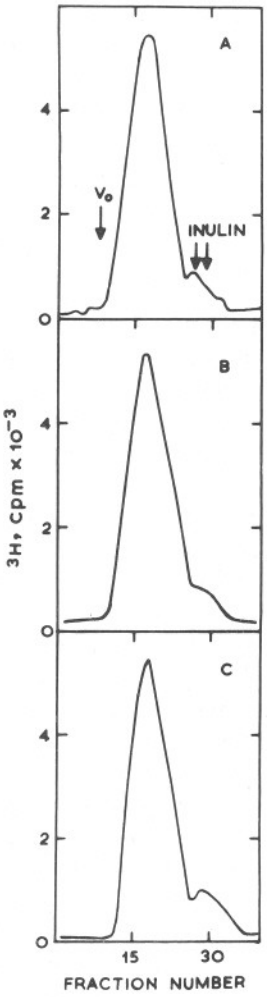

Fig. 3. Elution profile of phospholipase A_2 hydrolysed vesicles containing trapped [^{3}H]inulin. Vesicles containing trapped inulin were incubated separately with buffer (A), phospholipase A_2 (5 μg protein/μmol lipid P) and 10 mM Ca^{2+} (B), and 10 mM Ca^{2+} alone (C) at 25–30°C for 60 min. pH of the incubation mictures was 8.5. Measured aliquots of these incubation mixtures were chromatographed on Bio-Gel A-50m columns (1.2×15 cm). Each fraction (0.5 ml) was analysed by measuring total phosphorus and radio activity. Fractions eluted after the fraction number 28 did not contain phosphorus. Vesicles that did not receive the above treatments were also chromatographed on this column. The elution patterns of these vesicles were similar to that shown in the top panel (A). The small peak eluting like inulin is presumably of free inulin which did not separate at the initial purification step. Position of inulin was determined by passing free [^{3}H]inulin through the column.

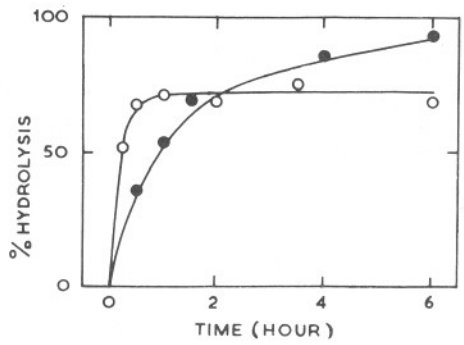

Fig. 4. Kinetics of hydrolysis of vesicle PC by phospholipase A_2. Vesicles containing trace amounts of egg [^{14}C]PC were treated, in the absence (○) as well as in the presence of 0.5% Triton X-100 (●), with phospholipase A_2 and the amounts of the hydrolysed PC were determined, as described in Materials and Methods.

due to partial cleavage of the external phospholipids because the amounts of degraded PC and PE in vesicles virtually remained constant between 1 h and 4 h after the enzyme treatments (Fig. 5).

In order to further examine the suitability of phospholipase A_2 to probe the phospholipid organization in the vesicles, we have studied the transbilayer distribution of aminophospholipids in the two selected types of vesicles (sonicated vesicles, with and without 40 weight % cholesterol) using the chemical probe, TNBS. The impermeability of the reagent across the bilayer was confirmed by studying the kinetics of amino group labeling in intact and lysed vesicles (Fig. 6). At all

TABLE III

VESICLE PHOSPHOLIPID DEGRADATION BY PHOSPHOLIPASE A_2

Values are mean of 4–6 determinations ± S.D.; PL, phospholipid; Chol, cholesterol (40 weight %).

Samples	PC (%)	PE (%)	PS (%)
Sonicated vesicles			
PL alone	73.4 ± 1.5	67.0 ± 1.4	46.5 ± 2.8
PL/Chol	70.2 ± 2.3	67.8 ± 1.1	55.6 ± 1.9
Detergent dialysed vesicles			
PL alone	62.6 ± 1.8	60.7 ± 2.5	54.8 ± 1.7
PL/Chol	59.0 ± 2.2	58.3 ± 2.4	57.4 ± 1.2

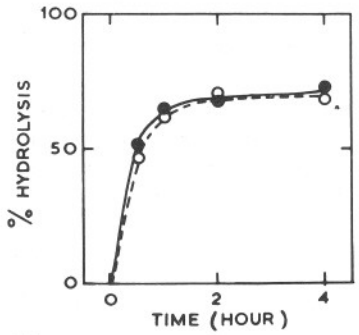

Fig. 5. Kinetics of phospholipase A_2 hydrolyses of PC and PE in vesicles. Phospholipase A_2 treatments of vesicles for different periods of time were carried out under the conditions as given in Materials and Methods. After blocking the enzyme activity with EDTA, the hydrolysed vesicle lipids were extracted and the amounts of the degraded phospholipids were determined. Values shown for PC (○) and PE (●) are mean from duplicate experiments. The variation was 2–5%. However, % degradation of PS at 30 min could not be determined with this accuracy. The amounts of hydrolysed PS in vesicles did not increase after the 2 h treatment.

time periods significantly higher amounts of the phospholipids were labeled by TNBS in the lysed vesicles as compared to those in the intact vesicles. A continuous increase in the labeling amounts

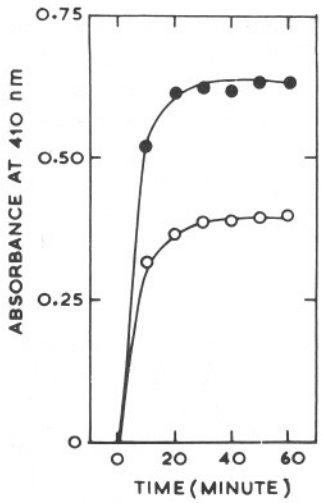

Fig. 6. Kinetics of labeling of the vesicles with TNBS at 20°C. Vesicles were treated with TNBS in the absence (○) as well as in the presence of Triton X-100 (●) as described in Materials and Methods. The amounts of amino group labelings at different periods of time were determined by measuring absorbance for yellow color at 410 nm, after acidification. In case when the same experiment was carried out at or above 25°C the labeling curve for intact vesicles did not plateau upto 60 min.

with time was observed upto the first 30 min. No further increases in the amounts occurred after this period. These findings indicate that under the present experimental conditions TNBS does not permeate the vesicle bilayer and thus the amounts of PE and PS that were labeled by this reagent in intact vesicles (without cholesterol: PE = 68 ± 2% and PS = 48 ± 3%; with cholesterol: PE = 66 ± 2% and PS = 54 ± 2%) should represent the total amounts of these phospholipids in the external monolayer. As may be seen in Table III, the amounts of PE and PS that were hydrolysed by phospholipase A_2 are quite comparable to those modified by TNBS in intact sonicated vesicles, again indicating that the enzyme degraded only the external phospholipids.

Several investigators have shown that the transbilayer exchange (flip-flop) of phospholipids in model membrane systems is a very slow process [22]. However, since rapid exchange can occur under some conditions [23], the possibility that inclusion of cholesterol in the phospholipid bilayers may enhance the transbilayer exchange was excluded by studying the kinetics of aminophospholipid labeling in the vesicles containing cholesterol. The results obtained in these studies were almost identical to that shown in Fig. 6, suggesting that cholesterol incorporation in vesicles did not induce phospholipid exchange across the bilayer.

These observations demonstrate that the phospholipase A_2 hydrolyses all the external glycerophospholipids in intact vesicles, and that there is no rapid transmembrane exchange of phospholipids across the vesicles bilayer. Thus the accessibility of the various phospholipids to the enzyme in intact vesicles can directly be correlated with their localization in the external monolayer. Hence, approx. 73% PC, 67% PE and 46% PS are distributed in the outer surface of the sonicated vesicles that were free of cholesterol (Table III). These amounts of the external glycerophospholipids account for only about 53% of the total vesicle phospholipids. As the fraction of phospholipid molecules in the outer monolayer of small vesicles is about two-third, approx. 70% of total vesicle SM should be external. This means that in these small vesicles PC, SM and PE are randomly distributed in the two monolayers, whereas PS is

localized preferentially in the inner monolayer. However, this asymmetric distribution of PS virtually disappeared in larger size vesicles (outer diameter, about 45 nm) that were formed by detergent dialysis and fractionated by centrifugation. Not only PS, but also PC, SM and PE were symmetrically distributed in the detergent dialysed vesicles (Table III), as the amount of external phospholipids in the vesicles having outer diameter about 45 nm is approx. 60% of the total vesicle phospholipids [24].

The observed transbilayer distributions of SM and PE in the sonicated vesicles do not seem to agree with the results of earlier studies which showed that PE in PC/PE (> 10 mol %) vesicles prefers the inner monolayer [4,5] and SM in PC/SM vesicles the outer monolayer [6]. This discrepancy is probably due to the marked differences between the lipid compositions of small vesicles studied here and previously [4–6]. However, as observed earlier in vesicles comprised of binary mixtures of phospholipids [6,7], PS in sonicated vesicles of erythrocyte membrane phospholipids distributed preferentially in the inner monolayer. Nevertheless, this asymmetric distribution of PS virtually disappeared in larger size vesicles, suggesting that the PS asymmetry in small vesicles was enforced mainly by the packing constraints imposed by the high degree of surface curvature [25]. It may therefore be inferred that erythrocyte membrane phospholipids in unilamellar vesicles prefer the random transbilayer distribution rather than the asymmetric distribution, as observed earlier in the native erythrocyte membrane [19].

Van Deenen and co-workers have shown that cholesterol, a major lipid component of red cell membrane, interacts preferentially with PC in PC/PE mixtures [26] and with SM in SM/PC mixtures [27]. To evaluate the influence, if any, of these differential interactions of cholesterol with phospholipids on the transbilayer distributions of the erythrocyte membrane phospholipids, we have analysed the phospholipid organization in vesicles consisting of the phospholipids and 40 weight % cholesterol. The effect of cholesterol on the vesicle asymmetry was ascertained by comparing the relative ratios of external glycerophospholipids. It was presumed that the higher affinity of cholesterol for SM and PC may, in principle, lead to a decrease in the amounts of external aminophospholipids and consequently an increase in the ratios of the external PC to the external PE (or PS). But Table IV shows that these ratios decreased upon incorporating cholesterol in either of the two types of vesicles, suggesting that the cholesterol incorporation leads to more random arrangements of phospholipids across the vesicles bilayer. This decrease in the ratios can not be due to preferential interactions of cholesterol with SM and PC but could arise from the cholesterol-induced increase in the size of vesicles. An increase in the vesicle size was quite evident from the elution profiles of cholesterol-containing vesicles from the Bio-Gel column, which is in agreement with earlier reports [28,29].

TABLE IV

RELATIVE RATIOS OF EXTERNAL GLYCEROPHOSPHOLIPIDS IN VESICLES

The ratios of the external PC ($[PC]_e$) to the external PE ($[PE]_e$) or the external PS ($[PS]_e$) were calculated from the mean value of the phospholipid fraction that was accessible to phospholipase A_2 in intact vesicles (see Table III). PL, phospholipid; Chol, cholesterol (40 weight %).

Samples	$[PC]_e/[PE]_e$	$[PC]_e/[PS]_e$
Sonicated vesicles		
PL alone	1.09	1.58
PL/Chol	1.03	1.26
Detergent dialysed vesicles		
PL alone	1.03	1.14
PL/Chol	1.01	1.03

Conclusion

Present study indicates that the transbilayer distributions of red cell membrane phospholipids in unilamellar vesicles are largely controlled by the packing constraints imposed by degree of surface curvature [25] rather than by interlipid interactions. The fact that the size of the red cell (about 8 μm) is much larger than that of the vesicles, these phospholipids in protein-free red cell membrane must be randomly distributed in the two monolayers. Therefore, the asymmetric distribution of phospholipids in this membrane should be induced by some factors other than the interlipid

interactions. We suggest that the phospholipid asymmetry in red cells primarily arises from differential bindings of phospholipids with membrane proteins in the two halves of the membrane bilayer. This suggestion finds strong support from the earlier studies which showed that red cell membrane skeletal proteins, especially spectrin and 4.1 polypeptide, differentially interact with phospholipids in monolayers, vesicles and erythrocyte ghosts [30–32]. That the interactions between the membrane skeletal proteins and the inner layer phospholipids probably maintain the phospholipid asymmetry in the native erythrocyte membrane is amply demonstrated in the studies of the mammalian red cells that possess abnormal membrane skeleton [33–37].

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