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# 1,2-Dotriacontanedioyl-*sn*-Glycero-3-Phosphocholine: A Cyclic Lipid Used for Phospholipase $A_2$-Catalyzed Modulation of the Liposome Surface Charge[1]

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**An aqueous suspension of liposomes, which were made of cyclic 1,2-dotriacontanedioyl-*sn*-glycero-3-phosphocholine (dTPC) and 1,2-diphytanyl-*sn*-glycero-3-phosphocholine, was incubatedwith phospholipase $A_2$ (*Naja mossambica mossambica*) in 0.05 M Tris-HCl, pH 7.7-7.8. At 25°C, approximately 30% of the cyclic lipids was digested into 1-($\omega$-carboxyhentriacontanoyl)-*sn*-glycero-3-phosphocholine (lyso-dTPC), the original morphology being preserved but with a decrease in the $\zeta$-potential of the membrane from *ca.* +2 to −1 mv. At 38°C in 30 min, as much as 45% of the cyclic lipids was attacked by the lipase, which resulted in lowering of the potential to −2.5 to −3 mV. The enzymatic surface-charge modulation was discussed in conjunction with a side-selective attack of the phospholipase on the lipid bilayer.**

**Key words: lipid bilayer, liposome, phospholipase $A_2$ (*Naja mossambica mossambica*), phosphatidylcholine, zeta-potential.**

Phospholipids are major constituents of many cellular membranes. Like other biomolecules, they are in a constant state of metabolic flux, which depends upon the physiological and pathological state of the organism (*1*). Few studies, however, have dealt with *in situ* modification of a lipid bilayer by artificial means. In the previous paper we showed that sonication of an aqueous suspension of cyclic 1,2-dotriacontanedioyl-*sn*-glycero-3-phosphocholine (dTPC; see Fig. 1 for lipid structure) furnished well-defined liposomes (*2*). The vesicles were similar to those prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in size, membrane thickness and gel-to-liquid phase transition temperature (42.6°C). dTPC was rather featured by the action of phospholipase $A_2$ ($PLA_2$); *viz.*, the lipid in the liposomes was converted readily into 1-($\omega$-carboxyhentriacontanoyl)-*sn*-glycero-3-phosphocholine (lyso-dTPC), which exposed anionic -$COO^-$ groups on the membrane surface. Although the electrosurface property of membranes may be altered on mixing with ionic lipids such as diacylglycero-3-phosphate and phosphatidylserine (*3, 4*) and possibly on the addition of phospholipase D, dTPC might be used as a tool for biological studies on lipid membranes taking advantage of its substrate-specificity. The present paper deals with our recent study on surface charge modulation by this cyclic lipid. 1,2-Diphytanyl-*sn*-glycero-3-phosphocholine (DPhyPC) was employed as a coexisting lipid because this isoprenoid lipid is not digested by $PLA_2$ but provides stable membranes (*5*).

## EXPERIMENTAL PROCEDURES

dTPC was synthesized and purified as described below. Lyso-dTPC and DPhyPC were prepared previously (*2, 5*). DPPC (purity at least 99%) was kindly donated by Nippon Fine Chemical. $PLA_2$ of *Naja mossambica mossambica* was purchased from Sigma, USA (product No. 7778). Sonication was performed with a Ohtake model 5201 ultrasonic disintegrator equipped with a titanium horn. Transmission electron micrographs were taken under a Hitachi H-7000 microscope. Light scattering spectra and the $\zeta$-potential were measured using a Ohtsuka ELS-800 spectrometer equipped with an electrophoresis cell.

*1,2-Dotriacontanedioyl-sn-Glycero-3-Phosphocholine (dTPC)*[3]—Anhydrous dotriacontanedioic acid (3.5 g, *ca.* 6.8 mmol) was stirred with thionyl chloride (20 ml, 0.28

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Abbreviations: CF, 5(6)-carboxyfluorescein; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPhyPC, 1,2-diphytanyl-*sn*-glycero-3-phosphocholine, where phytanyl is (3*RS*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl; dTPC, 1,2-dotriacontanedioyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid disodium salt; FABMS, fast atom bombardment mass spectroscopy; lyso-dTPC, 1-($\omega$-carboxyhentriacontanoyl)-*sn*-glycero-3-phosphocholine; $PLA_2$, phospholipase $A_2$ from *Naja mossambica mossambica*; $R_f$, relative mobility on TLC; TEM, transmission electron microscopy; TLC, thin layer chromatography.



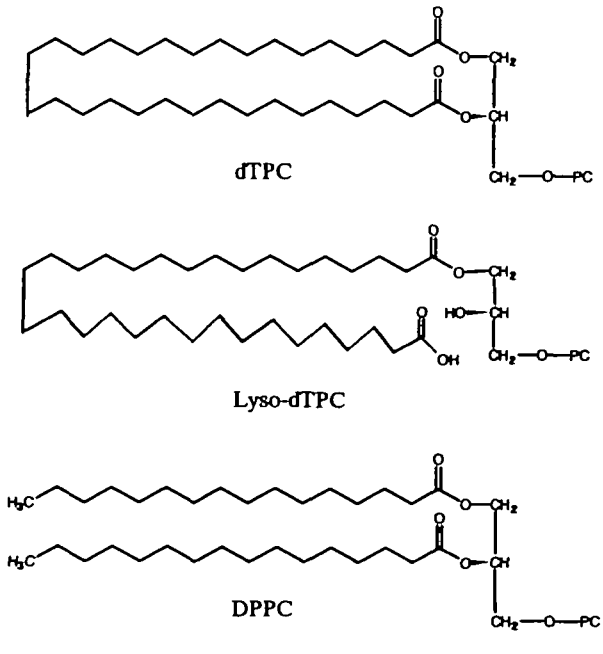
[3] In addition to dTPC, 1,26-hexacosanedioyl-*sn*-glycero-3-phosphocholine was isolated, 50 mg; yield, 1%; m.p. 140-144°C; $R_f$ 0.11 (chloroform-methanol-concentrated ammonia=65:35:5 v/v/v); IR (KBr) 2,900 (s), 1,740 (s), 1,460 (m), 1,240 (s), 1,160 (s), and 1,095 (s) $cm^{-1}$; FABMAS (matrix, glycerol) *m/e*, 676 (M+; relative intensity, 32). The by-product was derived most likely from the hexacosanedioic acid, a contaminant in the starting dotriacontanedioic acid.

mol) at ambient temperature for 24 h. Upon removal of light boiling materials from the reaction mixture under reduced pressure, the corresponding diacyl dichloride was obtained as a solid; 3.7 g; m.p. 64-67°C.

On the other hand, egg yolk phosphatidylcholine (approximately 60%; 40 g) was dissolved in diethyl ether (400 ml), mixed with a 25 wt% aqueous solution of tetrabutylammonium hydroxide (76 ml), and then stirred at room temperature for 2 h. The resulting precipitate was washed with the ether, dissolved in methanol, mixed well with Celite 535 (27 g), and then concentrated with a rotary evaporator to give *sn*-glycero-3-phosphocholine(GPC)/Celite as a powder (about 30 g).

To a mixture of the GPC/Celite (6.1 g) and 4-dimethylaminopyridine (2.0 g), which was suspended in anhydrous chloroform (30 ml) and cooled in an ice-water bath, was added dropwise a chloroform solution (about 10 ml) of the aforementioned dotriacontanedioyl dichloride. After sonication with a bath-type sonicator at about 50°C for 48 h, the reaction mixture was passed through a filter coated with Hyflo-Super-Cell (1 g) in order to remove Celite. The filtrate was concentrated and then applied to a silica gel column (Merck art. 7734, 7-340 mesh; 2.0 cm×20 cm). The column was developed with a mixture of chloroform, methanol and concentrated ammonia, the volume ratio of which was changed gradually from 65:15:2 to 65:35:5. The fraction which was positive on both Rhodamine 6G and Dittmer Lester-reagent spraying (*6*) was concentrated, and then applied to a Sephadex LH-20 gel column (2 cm×40 cm) with a mixture of chloroform and methanol (2:1 v/v) as the solvent.

The lipid fraction thus obtained was next applied to a preparative high pressure liquid chromatograph [column: TSK gel ODS-120T, 7.8 mm×300 mm; detector: reflective index-type; solvent, flow rate and pressure: chloroform-methanol-water (17:35:7 v/v), 3.4 ml/min, 65-70 kg/cm²] to give dTPC as an analytically pure compound; 75 mg (2% yield based on the starting GPC); m.p. 154-156°C; $R_f$ on TLC (Merck GF254 5735, type 60) 0.17 (chloroform-methanol-concentrated ammonia; 65:35:5 v/v/v); *c.f.*, DPPC exhibited a $R_f$ of 0.2 in the same solvent. The IR, 400 MHz $^1$H NMR, and FABMS spectra were similar to those reported previously (*2*).




where the PC group is —P(O)(O⁻)OCH₂CH₂N⁺(CH₃)₃.

Fig. 1. **dTPC, lyso-dTPC and DPPC.**

*Preparation of Aqueous Liposome Suspensions from dTPC and DPhyPC*—The cyclic dTPC (2.0 mg/ml) was dissolved in chloroform-methanol (2:1 v/v). A solution of DPhyPC (4.0 mg/ml) was prepared similarly. These solutions were mixed in various volume ratios, concentrated, and kept under reduced pressure overnight at ambient temperature. To the resulting various molar mixtures of dTPC and DPhyPC (total weight of the lipids, about 3.3 mg) was added 0.05 M Tris-HCl buffer (pH 7.8) containing 1.2 mM $CaCl_2$ (2 ml). After sonication at 50°C for 5 min×5 times at about 200 W per cm² of cross sectional area of the titanium tip, the resulting suspensions were centrifuged at 2,000×*g* for 15 min to afford the supernatant, which were subsequently applied to a gel chromatography column (1.7 cm×20 cm) packed with Sephadex G-50 using the same Tris-HCl buffer. The fraction (3 ml) containing liposomes was eluted as a front band containing more than 80% of the original total weight of the lipids. The aqueous suspension was employed in the next experiments.

*Action of $PLA_2$ on the Liposomes and Lipid Analysis*—To an aqueous suspension of liposomes (2.4:1.0 and1.6:1.0 mixtures of dTPC and DPhyPC) was added a stock solution (50 μl) of $PLA_2$, the stock solution being prepared by dissolving 1,500 units of the enzyme (1 mg protein) in 15 ml of distilled water. The glass tubes containing the assay solutions were shaken gently at 25°C. After 0 (before addition of the enzyme), 15, 30, and 45 min, 1 ml aliquots of the assay solution were processed with $NH_2OH/FeCl_3$ reagents according to the literature (*7*) in order to estimate the extent of hydrolysis of dTPC from the absorbance at 520 nm.

The lipid composition of the $PLA_2$-treated liposomes was analyzed as follows. A suspension was concentrated with a

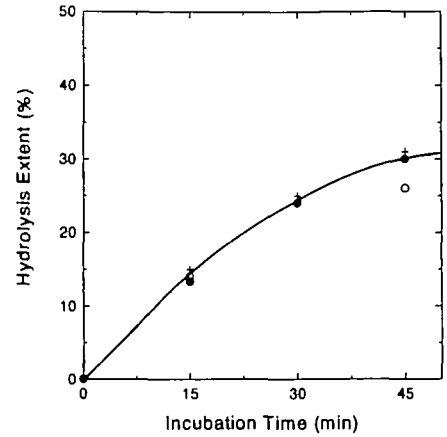



Fig. 2. **Phospholipase $A_2$-catalyzed hydrolysis of liposomal membranes made of a mixture of dTPC and DPhyPC at 25°C and pH 7.8 as a function of the incubation time.** +, ●, and ., dTPC/DPhyPC=1:0, 1:2, and 1:3 mol/mol, respectively.

rotary evaporator, dissolved in a mixture of chloroform and methanol (2:1 v/v), and then applied to a thin layer chromatograph sheet to determine the relative ratio of the phospholipids by Kates's micro-procedure (*8*). The mobilities of the phospholipids were identical with those reported previously (*2, 9*).

*Size Distribution and ζ-Potential of Liposomes*—An aqueous suspension of liposomes (dTPC:DPhyPC = 2:1 and 3:1 mol/mol; total amount, *ca.* 4.7 μmol) was treated with $PLA_2$ in a manner similar to that mentioned above. After incubation for 0, 15, 30, and 45 min at 25°C or 38°C, the assay solutions were each mixed with a saturated aqueous solution (50 μl or *ca.* 0.03 mmol) of EDTA in order to inactivate the enzyme, filtered through a cellulose acetate filter (pore size, 0.45 μm), and then placed in a quartz cell for light scattering measurement or a quartz cell for electrophoretic mobility (ζ-potential) measurement. EDTA adsorbed on lipid membranes would not significantly affect the potential since the total quantity of EDTA was much smaller than the combined amount of the lipids. Monodispersal liposome suspensions were obtained after the gel chromatography. The potential theory states that the magnitude is not a function of the vesicle size (*10*). Three experimental values were averaged.

*Transmission Electron Microscopy*—A half drop of each of the aqueous liposome suspensions, which were processed as above with or without $PLA_2$, was laid on a copper grid coated with a carbon/collodion film. A half drop of 1.5 wt% aqueous phosphotungstic acid/sodium hydroxide (pH 7) was then added to the solution on the grid, and staining was allowed to proceed at ambient temperature for 5 min. The excess liquid on the grid was removed with the corner of a piece of an absorbent paper, and the resulting specimen was mounted in an electron microscope to obtain images of the lipid assemblies at a magnification of $1\text{-}2\times10^4$. The pictures were usually enlarged by a factor of 5-10.

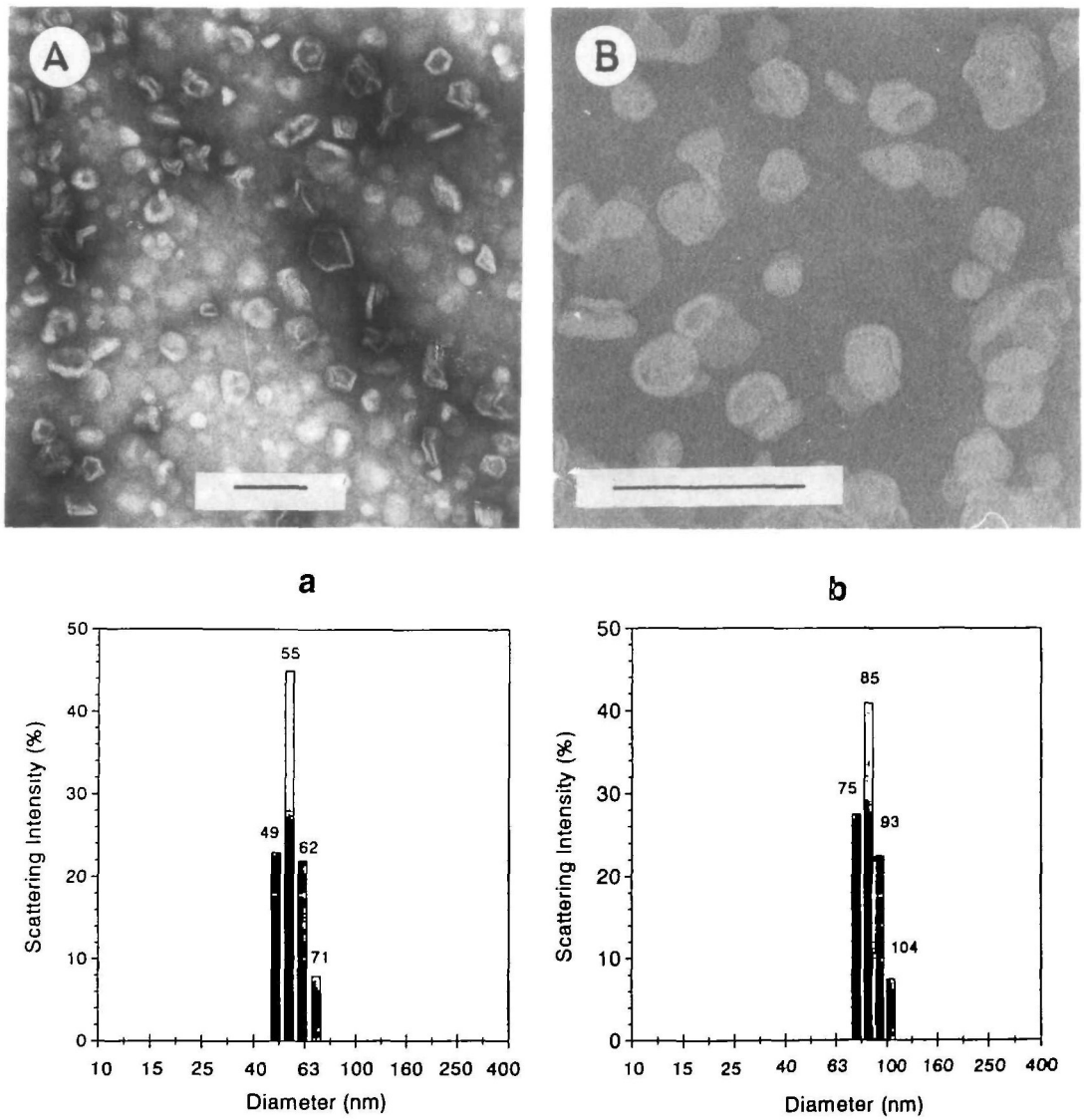



Fig. 3. **Transmission electron micrographs of liposomes comprising 1:3 molar mixture of dTPC and DPhyPC in 0.05 M Tris/HCl at 25°C; negatively stained with phosphotungustic acid/NaOH (pH 7).** A: before phospholipase $A_2$ treatment; B: after treatment for 30 min. The histograms (a and b for A and B, respectively) of the diameter distribution (number average) were obtained with a light scattering spectrometer. The diameter of the peaks is shown.

## RESULTS

*Action of $PLA_2$ on the Liposomes*—Figure 2 shows the time course of the extent of hydrolysis of dTPC through the action of $PLA_2$ on liposomes made of 1:0 (line —+—), 1:2 (line —●—), and 1:3 (line —○—) molar mixtures of dTPC and DPhyPC at 25°C and pH 7.8. The phospholipase only cleaved the *sn*-2-ester bond of the cyclic lipid to afford lyso-dTPC. The hydrolysis rate was not affected by the lipid composition. Vesicles with a hydrolysis-extent of 20-25% were somewhat larger in diameter than the original liposomes (85 *vs.* 55 nm), as revealed typically by the TEM pictures (Fig. 3, A and B) and light scattering measurements (Fig. 3, a and b), although 5(6)-carboxyfluorescein (CF), which had been trapped within the aqueous interior, was retained fairly well within the liposomes during the enzyme treatment (Fig. 4, solid bars for 25°C).

Incubation at 38°C, on the other hand, enhanced the hydrolysis to an extent of 85-95% within 15 min. CF escaped rapidly from any liposomes into the outer aqueous phase (Fig. 4, shadowed bars). TEM suggested that the vesicular morphology was preserved only when the molar ratio of dTPC:DPhyPC was 1:10 and perhaps higher.

*Effect of $PLA_2$-Action on the $\zeta$-Potential of Liposomes*—The time-course of the $\zeta$-potential at 25°C shown in Fig. 5. The potential decreased in a similar manner at 38°C. The $\zeta$-potential of $PLA_2$-untreated and -treated liposomes was more negative when the relative ratio of dTPC (to DPhyPC) was larger; for instance, see Fig. 5 (line —●—). This phenomenon might be due to the relatively large surface area of DPhyPC in the membranes; *viz.*, *ca.* 50 Å² of dTPC (*1*) *vs. ca.* 100 Å² of DPhyPC (*11*). The phospholipase (the total amount added to the liposome suspension, 3.3 $\mu$g) adsorbed at the interface (total amount of lipids, 3 mg) may not significantly affect the $\zeta$-potential because most of the lipase molecules are considered to exist in the aqueous phase; *c.f.*, the "DISCUSSION" section.

## DISCUSSION

This cyclic dTPC would be considered as a biologically inactive lipid, being similar to ordinary double chain-phosphatidylcholines such as DPPC. Through the action of phospholipase $A_2$ ($PLA_2$), however, the isoelectrically neutral lipid changes suddenly into lyso-dTPC which, unlike ordinary lysolipids, does not disrupt the membrane structure. The present paper deals mainly with the *in situ* change of the $\zeta$-potential of liposomes during incubation in the presence of the phospholipase. For this, DPhyPC was chosen as a coexisting lipid because (i) a mixture of the archaebacterial type lipid with various phosphatidylcholines had been shown to provide highly stable liposomes (*5, 12*) and (ii) DPhyPC is neither a substrate nor an inhibitor of the $PLA_2$.

Therefore, the action of the lipase at 25°C allowed the hydrolysis to proceed to 25-35% extent, as shown in Fig. 2. The hydrolysis-rate was not affected by the ratio of the lipids in the liposomes (dTPC:DPhyPC=1:0, 1:2, and 1:3 mole ratio). The $\zeta$-potential of the liposomes gradually leveled off, as can be seen in Fig. 5, and the higher the original ratio of dTPC/DPhyPC was the lower the potential of both original and digested vesicles was, because of the large limited area occupied by DphYPC molecules (lines ○ *vs.* and ●). When the mole ratio of dTPC/DphYPC was less than 1/2, the enzymatic hydrolysis did not significantly disturb the original morphology of the liposomes, although the diameter (75-104 nm; mean size: 85 nm) became somewhat larger than that (49-71 nm; mean size: 55 nm) of the intact vesicles (Fig. 3).

In contrast, the action of $PLA_2$ at 38°C in 30 min resulted in the hydrolysis of as much as 45% of the dTPC molecules. Under the assay condition used, the $\zeta$-potential decreased to −2.5 to −3 mV. The treatment also resulted in the release of most CF molecules from the interior of the vesicles (Fig. 4, shadowed bars). This phenomenon may be ascribed to the membrane deformation due to the extensive

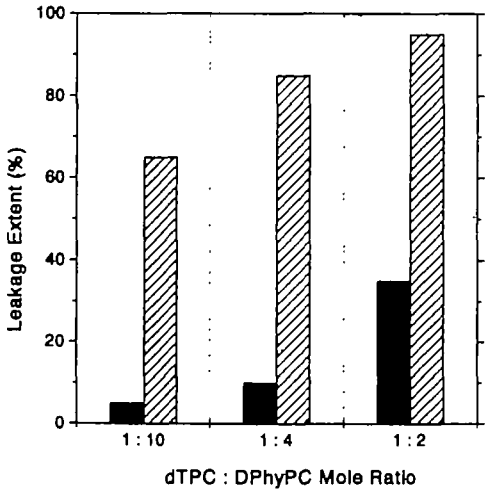



Fig. 4. **Effect of the phospholipase $A_2$ action on the extent of leakage of CF from the aqueous interior of liposomes with various dTPC/DPhyPC mole ratios.** Black and shadowed bars, treatment at 25°C for 30 min and 38°C for 30 min, respectively.

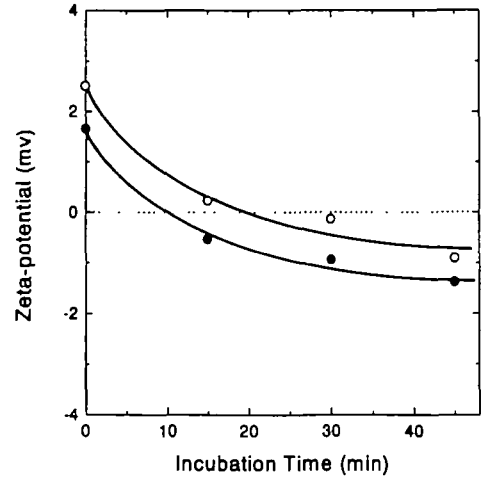



Fig. 5. **Effect of the phospholipase $A_2$ action on the $\zeta$-potential of liposomes made of a mixture of dTPC and DPhyPC as a function of the incubation time.** ● and ○, 1:2 and 1:3 molar mixtures of dTPC:DPhyPC at 25°C in 0.05 M Tris-HCl (pH 7.8), respectively.

hydrolysis and to the assay temperature, which was close to the phase transition temperature (42°C) of the dTPC bilayer itself.

It may be considered that the $PLA_2$ modified the liposome surface in the following way. The enzyme molecules, upon addition to an aqueous suspension of vesicles, diffused into and then settled on the outer interface of the liposomes, presumably orienting the interfacial recognition site to the membrane; *c.f.*, the concentration ratio (interface/bulk aqueous phase) of the enzyme was estimated to be about 18 at 25°C (*12*). Here, it appears that the catalytic site of the $PLA_2$ (*13, 14*) does not recognize the hydrophobic moieties of lipids since the cyclic dTPC was almost the same as DPPC in substrate specificity (*2, 9*). At 25°C the liposomes comprising a 1:3 molar mixture of DPPC and DPhyPC well retained the carboxyfluorescein-$PLA_2$ conjugate in their aqueous interior; *c.f*, the assay was conducted in the presence of EDTA, an inhibitor of $PLA_2$. Therefore, it would be considered that, since the $PLA_2$ was added to the outside region of the liposome suspensions, the dTPC molecules in the outer half of the bilayer were digested preferentially (to the lipids in the inner half). The developing negative potential is unlikely to affect the enzyme action (*15-17*). The present hydrolysis is in accord in part with previous reports on the side selective hydrolysis of liposomes, erythrocytes and bacterial membranes by $PLA_2$ at 25°C (*18-21*).

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