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Surface charge modulation of liposomes by enzymatic hydrolysis of macrocyclic 1,2-dotriacontanedioyl-*sn*-glycero-3-phosphocholine

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1,2-Dotriacontanedioyl-*sn*-glycero-3-phosphocholine (dTPC) was synthesized, and by a sonication method, dTPC was transformed into liposomes with physical features (charge, size, gel-to-liquid crystalline phase transition constants, etc.) similar to those of liposomes made of acyclic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). Quantitative enzymatic assays using phospholipases A_2 and C showed that dTPC was comparable with or better than DPPC as a substrate. Remarkably, the liposomes assembled from a mixture of dTPC and 1,2-distearyl-*sn*-glycero-3-phosphocholine were converted rapidly into anionic liposomes at pH 7 by the action of phospholipase A_2, keeping their vesicular structure and exposing CO_2H groups of the lysolipids of dTPC on the membrane surface. The use of dTPC is discussed in conjunction with the enzyme-catalyzed modification of the liposomes.

Introduction

Recent interest in lipids as biomembrane-forming materials has led to the synthesis of various artificial amphiphiles [1]. These compounds may be completely different in structure from ordinary cytoplasmic lipids, and they simulate only the physicochemical properties of biomembranes [2–5]. But if one aims also to apply the amphiphiles to a living system [6,7], an ideal compound may possess the following properties: (a) it can form liposomes or other membranous structures by itself or in cooperation with cytoplasmic lipids; (b) it disturbs neither the physicochemical properties (surface charge, thickness, fluidity and lipophilicity) nor the biological functions (transports, biosyntheses, etc.) of the native plasma membranes, (c) the dormant amphiphile is, however, rendered active upon biotransformation in the system.

In this paper, we wish to describe such a potential lipid, 1,2-dotriacontanedioyl-*sn*-glycero-3-phosphocholine (dTPC). We first deal with the synthesis of dTPC and the physicochemical properties of the liposomes. The second part is concerned with enzymatic susceptibilities of the macrocyclic lipid to phospholipases in comparison with those of natural 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). Plausible biological uses of dTPC will be discussed in the subsequent section.

Abbreviations: dTPC, 1,2dotriacontanedioyl-*sn*-glycero-3-phosphocholine; DPPC, acyclic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; FAB, fast atom bombardment; HPLC, high-pressure liquid chromatography; T_c, gel-to-liquid crystalline phase transition temperature.

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Materials and Methods

Materials and Instruments

DPPC (purity, at least 99%) was kindly donated by Nippon Fine Chemical Co., 1,2-distearyl-*sn*-glycero-3-phosphocholine (DSPC) was prepared previously [8]. Phospholipase A_2 of *Naja naja* and phospholipase C of *C. perfringens* were purchased from Sigma, U.S.A. (product No. 7633 and 7778, respectively). Thin-layer chromatography (TLC) was carried out on silica-gel-precoated sheet (Merck Art. 5735) using solvent systems described elsewhere. Compound spots were visualized under ultraviolet light spraying with 0.0012% aqueous Rhodamine 6G (for the long-chain acyl group) [9] and the Dittmer-Lester reagent (for phosphoric acid ester) [10]. Column chromatography was performed using a glass column packed with silica gel (Merck 7734, 70-230 mesh). High-pressure liquid chromatography (HPLC) was conducted using a Toyo-soda 803 chromatograph packed with silica gel (TSKgel ODS-120T) in a stainless-steel column (7.8 mm × 30 cm). The lipid peaks were monitored by means of a Shimazu RID-2A refractive index detector. ^{1}H-NMR spectra were measured on JEOL PS-100 and JEOL GX-400 spectrometers using a dilute solution in C^2HCl_3 or $C^2HCl_3/C^2H_3O^2H$ (2 : 1, v/v). Fast atom bombardment (FAB) mass spectra were measured using a JEOL HX-100, whereby a sample was mixed with glycerol or triethanolamine on a standard FAB target, and then subjected to a beam of xenon atoms produced at 8 kV and 2 mA. Ultrasonication was performed by the use of a probe-type ultrasonic disintegrator (Ohtake Works Co, model 5201). Transmission electron-microscopic migrographs were taken using a Hitachi H-300 microscope. Phase-transition temperature, zeta-(ζ-) potential and weight-average diameter of liposomes were determined by means of a differential scanning calorimeter (Rigaku, DSC-8230), a particle electrophoresis apparatus (Rand Brother, model 1, Mark II equipped with a halogen lamp) and a dynamic light scattering spectrometer (Ohtsuka DLS-700), respectively.

Preparation of dTPC

To a vigorously stirred suspension of a dry mixture of *sn*-glycro-3-phosphocholine (1.26 g, 5 mmol) and Celite (No. 535, Johns-Manville) (5.7 g) in dry chloroform cooled in an ice/water bath, was added 4-dimethylaminopyridine (1.2 g, 10 mmol), followed by a chloroform solution (10 ml) of 1,32-dotriacontanedioyl dichloride (2.8 g, 5 mmol). The reaction mixture was stirred at room temperature for 48 h and then concentrated under reduced pressure to give the residue, which was treated with a short silica-gel column (2 cm × 10 cm) using chloroform/methanol/water (65 : 35 : 5, v/v). The fraction, which gave a TLC-spot at R_F = approx. 0.35 (solvent, chloroform/methanol/conc. ammonia, 65 : 35 : 5, v/v) upon spraying with the Rhodamine 6G and the Dittmer-Lester reagent, was concentrated and rechromatographed on Sephadex LH-20 column (2.5 × 60 cm) using chloroform/methanol (2 : 1, v/v). The resulting almost pure dTPC was finally treated with a semipreparative HPLC (solvent, chloroform/methanol/water (17 : 35 : 5, v/v); flow rate, 2.8 ml/min; pressure, 70 kg/cm^2; temperature, approx. 25 °C) to give the lipid as an analytically pure compound with an elution time of approx. 11 min; yield, 0.29 g (8%); m.p., 240–242 °C; $[\alpha]_D^{20}$ + 5.8° (*c*, 0.6, chloroform); 400 MHz ^{1}H-NMR ($C^2HCl_3/C^2H_3O^2H$, 2 : 1, v/v) δ 1.285 (coherent peak, 52, $(CH_2)_{26}$), 1.608 (m, 4, 2 × CH_2), 2.317 and 2.336 (t, 2, J = 7.9 Hz, *sn*-1- or -2-CH_2), 3.221 [s, 9, $N(CH_3)_3$], and 5.258 (m, 1, *sn*-2-CH); FAB mass spectrum (Xe; matrix: triethanolamine), *m/z* (rel. intensity) 732 (*M*, 75); mobility in silica-gel TLC (Merck precoated sheet 5735, type 60; chloroform/methanol/conc. ammonia (65 : 35 : 5, v/v), 0.34. Analysis for $C_{40}H_{78}NO_8P$:
Calcd.: C, 65.63; H, 10.74; N, 1.91.
Found: C, 65.28; H, 11.10; N, 1.76.
As described elsewhere, dTPC was hydrolyzed completely by the phospholipase A_2 and the $[\alpha]_D^{20}$ value was very close to that of DPPC, $[\alpha]_D^{20}$ + 6.6° (*c*, 2.0 chloroform). A configuration of the *sn*-2 position of dTPC was hence assigned as the same as that of the starting *sn*-glycero-3-phosphocholine.

Preparation of liposomes

The lipid (dTPC, DPPC or DSPC), or a mixture of the lipids (the mole ratio is described elsewhere in the text) (10 mg unless specified) in 5 ml of distilled water or 0.05 M Tris-HCl buffer

(pH 7.2) was vortexed for a few minutes. The sample was then sonicated at 50 W, at 55°C for 5–15 min. The resulting solution was centrifuged at approx. $3000 \times g$ for 30 min. The supernatant was then subjected to electron-microscopic observation, measurement of physical constants (T_c, ζ-potential, etc.) and/or enzymatic assays.

Liposome structure

A half drop of the sonicated solution prepared as above was laid on a copper grid which was coated with a carbon film of approx. 200 Å thickness. A half drop of 2% (w/v) aqueous uranium acetate was then added to the solution on the grid, and staining was allowed to proceed at room temperature for 20 min, or at 35–40°C for a few minutes (for a sonicated solution of dTPC). The excess liquid on the grid was removed by means of the tip of an adsorbing paper, and the resulting specimen was mounted onto the electron microscope to observe the image of the liposomes at a magnification of 10^4 or $2 \cdot 10^4$. The pictures were usually enlarged by a factor of 10. The phospholipase A_2-treated vesicle solutions (vide infra) were also processed in a manner similar to that mentioned above, to examine the effects of hydrolysis of dTPC on the shape of the vesicles. Figs. 1a and b and 5a–c are typical microscopic observations.

Differential scanning calorimetry of the liposomes

About 10 mg of a lipid (DPPC, dTPC) or a 1 : 1 molar mixture of DPPC and dTPC in pure water (1 ml) was vortexed and sonicated as mentioned above. The vesicle solution (about 30 mg) was weighed accurately and placed in an aluminum pan and subjected to the measurements of endothermic T_c and ΔH values by means of the highly sensitive calorimeter. Each sample was measured three times to obtain the average values; experimental error: T_c, ± 0.5°C; ΔH, ± 0.3 kcal/mol. A reference pan contained approximately the same weight of pure water.

ζ-Potential and vesicle diameter of phospholipase A_2; treated and untreated liposomes of a mixture of dTPC and DSPC

16 ml dTPC (2 mg, 2.7 mmol)/DSPC (10 mg, 13 mmol)/0.05 M Tris-HCl buffer (pH 7.2) was sonicated to obtain a solution of the mixed liposomes, which was freezed subsequently in a methanol/solid CO_2 bath, then melted slowly at ambient temperature. The resulting solution was treated in a bath-type sonicator (Branson model B 12) at 55°C, at 40 W until the solution became transparent, then centrifuged at approx. $3000 \times g$ for 15 min. The supernatant, containing large unilamellar vesicles, was divided into two equal portions in test-tubes, and placed in a water-bath at 42°C. The stock solution of phospholipase A_2 (50 μl, vide infra) was added to one of the same solutions. After 30 min, both enzyme-untreated and enzyme-treated solutions were subjected to measurements of (a) weighted average diameter of the liposomes by means of the dynamic light-scattering spectrometer at an angle of 90° and at 25.6°C and (b) ζ-potentials by means of the particle electrophoresis apparatus at 25°C. As mentioned below, the incubation time of 30 min was long enough to convert almost all dTPC molecules in the mixed liposomes into the lysolipid (**3**).

Enzyme assays

The literature procedures were modified [11,12].

(1) Phospholipase C. A lipid (dTPC or DPPC, 3.0 mg) in 0.15 M NaCl (1.5 ml) was sonicated and centrifuged to give the supernatant as a substrate solution. The phospholipase C (2.8 mg) was dissolved in 6 ml 0.002 M $CaCl_2$/0.006 M imidazole-HCl buffer (pH 7.0), and the enzyme solution (1.5 ml) was pipetted into a test-tube which was kept in a water-bath thermostated at 42 ± 0.3°C. The above substrate solution was then added to the enzyme solution and the mixture was stirred gently. At incubation times of 7, 15, 23 and 30 min, an aliquot (0.1 ml) of the assay solution was mixed with 5 drops of 0.2 M EDTA to quench the hydrolysis, then spotted onto a silica-gel TLC plate, which was then developed with chloroform/methanol/conc. ammonia (6 : 35 : 5, v/v), to exhibit the spots of the starting lipids and the products (R_F); dTPC (0.34), DPPC (0.45), 1,2-dotriacontanedioyl-*sn*-glycerol (**1**, approx. 1), 1,2-dipalmitoyl-*sn*-glycerol (**2**, approx. 1) and phosphorylcholine (0). The starting lipid spot was cut from the plate and its phosphorus content was quantitated according to a described procedure [13]. The values were calibrated by subtracting the

value of a blank spot, and the % hydrolysis was calculated by the equation

$$100 \times \frac{\text{(observed phosphorous value)}}{\text{(control run value)}}$$

(the control run refers to the original substrate solution). Similarly, the enzyme assays were conducted at 33 and 51°C. The representative results are shown in Fig. 2a–c. Each spot in the figures represents an average of three runs. The reproducibility of the extent of hydrolysis was about ±2.5%.

To each of the remaining assay solutions was added diethyl ether (2.0 ml) after 4 h of incubation, and the mixture was stirred magnetically for 20 min. The resulting aqueous and organic phases were then analyzed for the hydrolysis products in a manner similar to that mentioned above. The results are displayed in the broken lines in the figure.

In contrast, the liposome solutions made of only dTPC or DPPC were also treated by phospholipase C in order to identify the products. Namely, each enzyme-treated solution was concentrated and extracted with diethyl ether. The organic extract was then applied to a silica-gel column. Elution with chloroform/1-propanol (20 : 1, v/v) gave the corresponding diacylglycerol in the yield of 50–60%.

1,2-Didotriacontanedioyl-*sn*-glycerol (**1**): m.p. 75–77°C; R_F (chloroform/1-propanol, 20 : 1, v/v); IR (KBr) 3400(s), 2920(s), 2840(m), 1735(s), 1460(m), 1375(s) and 1080(s) cm^{-1}; 100 MHz ^{1}H-NMR (C^2HCl_3) δ 1.24 (coherent s, 52H, $(CH_2)_{26}$), 1.58 (broad, 4H, $2 \times \beta CH_2$), 2.15 (s, 1H, OH), 2.30 and 2.32 (t, 2H, $J = 7.2$ Hz, α- or $\alpha' CH_2$), 3.73 (broad d, 2H, $J \approx 5$ Hz, CH_2OH), 4.24 (complex m, 2, CH_2OCO), 5.06 (quintet, 1H, $J \approx 5$ Hz, CH); field desorption mass spectrum, 566 (calculated for $C_{35}H_{66}O_5$, 566.9).

1,2-Dipalmitoyl-*sn*-glycerol (**2**) was identified by comparison of the IR and ^{1}H-NMR spectra as well as the R_F value (0.71 by chloroform/1-propanol, 20 : 1, v/v) with those of an authentic sample prepared previously [14].

(2) Phospholipase A_2. 12 ml dTPC (6.0 mg)/DPPC (6.0 mg)/0.05 M Tris-HCl buffer (pH 7.2) was sonicated and centrifuged to furnish the supernatant as a substrate solution. The phospholipase A_2 (0.6 mg) was dissolved in the same buffer (5 ml), and 50 μl of the enzyme stock solution was added to the substrate solution (2.0 ml), which was kept at a suitable temperature (32, 37, 42, 47 or 52°C ± 0.3°C) with gentle stirring. After incubating for 0 (a control in the absence of the enzyme), 3, 12 or 28 min, 0.4 ml of each assay solution was acidified by addition of one drop of concentrated hydrochloric acid to quench the reaction, and concentrated by means of a rotary oil pump. The resulting residue was dissolved in a small amount of chloroform, and the whole solution was spotted on the silica gel TLC plate. The plate was then developed using chloroform/methanol/conc. ammonia (65 : 35 : 5, v/v) to exhibit the spot of the starting lipids (mobility (R_F): dTPC. 0.35; DPPC, 0.43), 1-(ω-carboxyhentriacontanoyl-*sn*-glycero-3-phosphocholine (**3**, 0.04), 1-palmitoyl-*sn*-glycero-3-phosphocholine (**4**, 0.13) and palmitoic acid (0.68). Each spot of the unreacted lipids was cut from the plate and its phosphorus content was determined quantitatively according to a described method [13]. The extent of hydrolysis at each reaction time was calculated on the basis of the control. Similarly, a phospholipase A_2 assay was conducted for a sonicated solution of DPPC in order to check the effects of the lipid on the hydrolysis of the aforementioned liposome bilayers made of a mixture of dTPC and DPPC. Typical results are shown in Figs. 3 and 4.

In addition, all of the phospholipase A_2-assayed solutions were combined and concentrated, to yield a residue which was agitated with chloroform/methanol (2 : 1, v/v) (10 ml). After removal of undissolved material, the organic solution was concentrated and applied to a semipreparative HPLC, employing the same conditions as those for purification of dTPC (vide supra). The lysolipid (**3**) was eluted with a retention time of approx. 4 min; m.p., 147–155°C; $[\alpha]_D^{20}$ -2.0° (*c* 0.2, chloroform/methanol, 2 : 1, v/v); 400 MHz ^{1}H-NMR ($C^2HCl_3/C^2H_3O^2H$, 2 : 1, v/v) δ 1.269 (coherent peak, 52, $(CH_2)_{26}$), 1.618 (m, 4, $2 \times \beta CH_2$), 2.276 (t, 2, $J = 7.5$ Hz, CH_2CO_2H), 2.354 (t, 2, $J = 7.8$ Hz, CH_2OCOCH_2), and 3.218 (s, 9, $N(CH_3)_3$); FAB mass spectrum (Xe; matrix: glycerol), *m/z* (rel. intensity) 750 (*M*, 5); mobility in silica gel TLC (the plate and the solvent are the same as

those used for dTPC) 0.03–0.04. Analysis for $C_{40}H_{80}NO_9P$:
Calcd.: C, 64.08; H, 10.76; N, 1.88.
Found: C, 63.85; H, 11.12; N, 1.62.

Results

dTPC

A reaction of *sn*-glycero-3-phosphocholine and 1,32-dotriacontanedioyl dichloride produced dTPC in the yield of 8–15%. IR and ^{1}H-NMR spectra of dTPC were very similar to those of DPPC, except for the absence of two triplet NMR-signals for the terminal methyl-groups of DPPC. The polarity of the cyclic dTPC appeared to be slightly higher than DPPC as judged from the mobility in a silica-gel TLC, namely, dTPC, 0.34, DPPC, 0.40 (solvent: chloroform/methanol/conc. ammonia, 65 : 35 : 5, v/v).

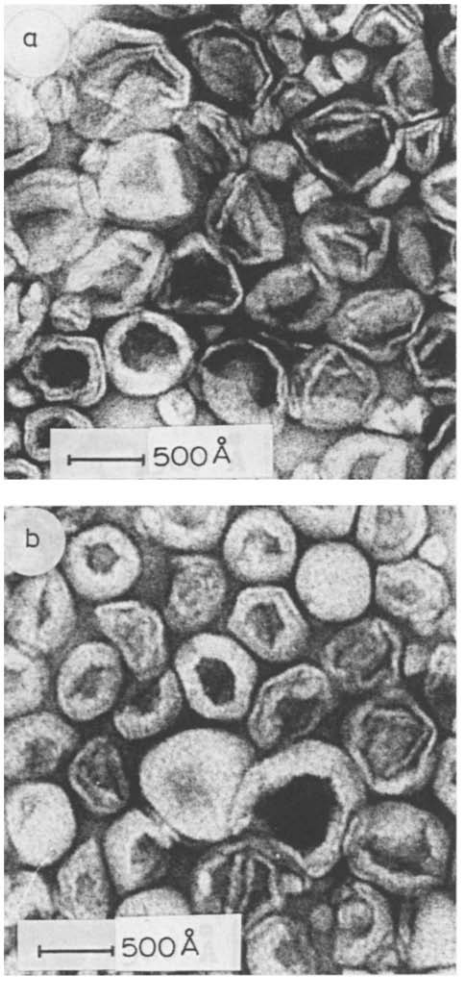


Fig. 1. Electron micrograpahs of liposomes negatively stained with uranyl acetate. (a) Aqueous solution of dTPC (2.7 mmol/l) sonicated at 55 °C, at 50 W for 10 min. (b) Aqueous solution of a 1 : 1 molar mixture of dTPC and DPPC (total 2.7 mmol/l) prepared under sonicating condition similar to those of (a).

Liposomes: structure and differential scanning calorimetry

When an aqueous suspension of dTPC was sonicated at 55 °C, at 50 W for 10 min, the lipid formed small uni- and oligolamellar vesicles with a diameter ranging from 200–800 Å (Fig. 1a). The small vesicles, (this term is used hereafter), were similar in size and shape to the liposomes made of DPPC under the same conditions. Here it was noted that, while the sonicated solution of DPPC was stable even after 10 days or more, the sonicated solution of dTPC was relatively unstable, and turned into a turbid solution after having stood at room temperature for several hours. The stability of liposomes containing dTPC, however, appeared to be improved by sonicating dTPC in the presence of more than 1 equiv. DPPC (Fig. 1b). A differential scanning calorimetry of the aforementioned liposome solutions gave the following endothermic gel-to-liquid crystalline phase transition temperatures, T_c (peak-width at half height), and transition enthalpies, ΔH; dTPC: 42.6 °C (1.0 °C), −5.2 kcal/mol; DPPC; 41.2 °C (1.5 °C), −7.3 kcal/mol; a 1 : 1 molar mixture of dTPC and DPPC: 42.1 °C (approx. 3 °C), −6.7 kcal/mol.

Phospholipase C-catalyzed hydrolysis of dTPC

Treatment of the sonicated solution of dTPC (small vesicles) with the enzyme at pH 7.0 readily hydrolyzed the macrocyclic lipid to liberate **1** and phosphorylcholine. Interestingly, the hydrolysis of dTPC occurred twice as fast as that of DPPC at 42 °C ($= T_c$) as seen in the initial slope (the reaction rate) of the extent of the hydrolysis vs. time-curve in Fig. 2b. Furthermore, while the enzyme decomposed dTPC efficiently to approx. 95% completion after 15–20 min at the temperature, the hydrolysis of DPPC reached an equilibrium state with only 75–80% conversion into **2** and phosphorylcholine. A similar trend was

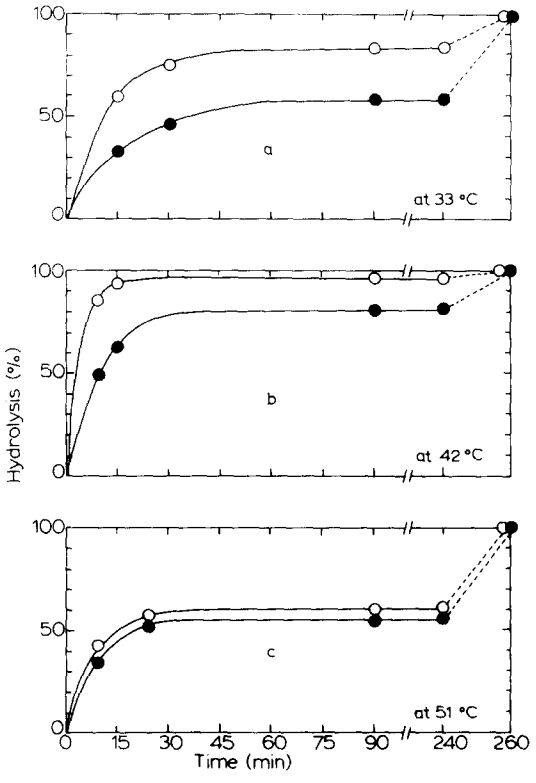


Fig. 2. Plot of the extent of phospholipase C-catalyzed hydrolysis (%) of dTPC (○) and DPPC (●) at specified temperature and pH 7.0, as a function of time. Assay solutions: small vesicle solution of the lipid (3.0 mg) in 0.15 M NaCl (1.5 ml) plus solution of phospholipase C (0.7 mg) in 0.006 M imidazole HCl buffer (pH 7.0) containing 0.002 M $CaCl_2$ (1.5 ml).

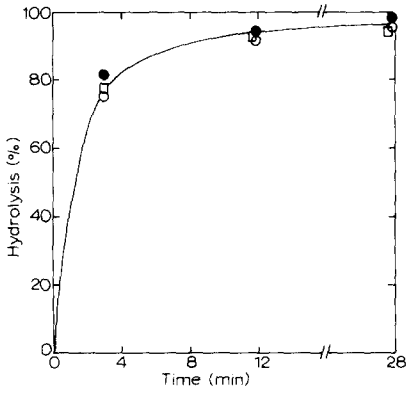


Fig. 3. Plot of the extent of phospholipase A_2-catalyzed hydrolysis (%) of DPPC (□) in the lipsomes, and of dTPC (○) and DPPC (●) in the liposomes made of a 1 : 1 molar mixture of the lipids at 42 ° C and pH 7.2, as a function of time. Assay solution: small vesicle solution (2.0 ml) of DPPC (2.0 mg), and small vesicle solution (2.0 ml) of dTPC (1.0 mg) and DPPC (1.0 mg) in 0.05 M Tris-HCl buffer (pH 7.2) plus phospholipase A_2 (6 μg) dissolved in the same buffer (50 μl).

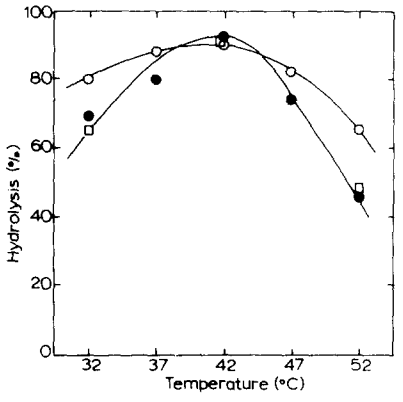


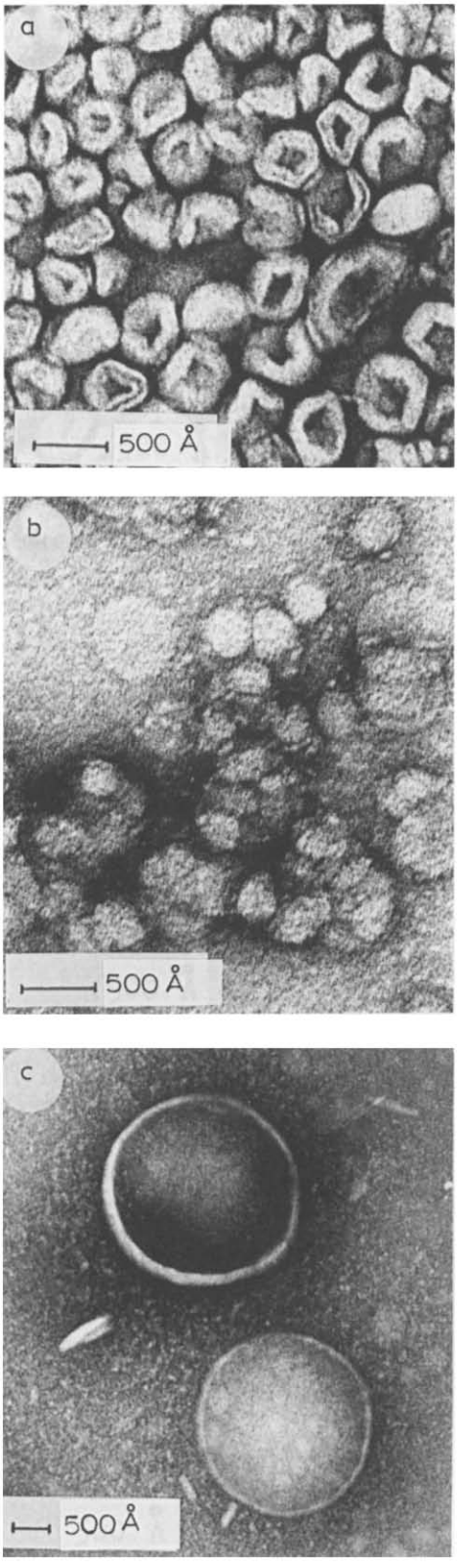
Fig. 4. Plot of the extent of phospholipase A_2-catalyzed hydrolysis (%) of DPPC (□) in the liposomes, and dTPC (○) and DPPC (●) in the liposomes made of a 1 : 1 molar mixture of the lipids at pH 7.2 for 12 min, as a function of temperature. Assay solutions were the same as those mentioned in the legend of Fig. 3.

observed at lower (33 ° C) and higher (51 ° C) temperatures than T_c (Fig. 2a and 2c). However, upon addition of diethyl ether to the assay solutions of the equilibrium state, both kinds of lipid were hydrolyzed rapidly and completely, as shown in the broken lines in Fig. 2a–c.

Phospholipase A_2-catalyzed hydrolysis of dTPC

Action of the enzyme on the sonicated solution yielded the peculiar lysolipid (3). Quantitative enzymatic assays at pH 7.2 using the sonicated solution of DPPC and a 1 : 1 molar mixture of dTPC and DPPC (Fig. 1b) revealed that the rates of hydrolysis of both kinds of lipid in the mixed liposomes were very similar to each other, and DPPC did not affect the hydrolysis of dTPC (Fig. 3 and 4) *. An optimal temperature (approx. 40 ° C at pH 7.2, cf. Fig. 4) for the phospholipase A_2-

* We did not observe the 50% hydrolysis extents as shown in Figs. 3 and 4, although the phenomenon was reported by Wilschut et al. [19] as the result of selective degradation of the outer leaflet of the liposomes, which were made of a 1 : 1 molar mixture of egg-yolk phosphatidylcholine and phosphatidylethanolamine, with phospholipase A_2 (bee venom). We did, however, observe approx. 50% hydrolysis when pH values of the inner and outer (bulk) domains of small liposomes made of either dTPC or DPPC were adjusted to 7 and 3, respectively, when phospholipase A_2 (*Naja naja*) was added to the liposome solutions. These peculiar differences are currently under investigation.



catalyzed hydrolysis of dTPC agreed with the T_c (42.6 °C) determined by means of differential scanning calorimetry. It was also found that the small vesicles made of a mixture of dTPC and DSPC (mole ratio, 1: > 1) retained its liposome structure, even when all of dTPC in the liposomes was hydrolysed into **3** upon a treatment with phospholipase A_2 over the temperature range examined (25–55 °C); by contrast, the liposomes of a mixture of DPPC and DSPC (the more ratio examined, 1 : 5 and 1 : 10) were destroyed with a few minutes by the action of the enzyme. Typical examples are shown in Fig. 5a and b.

ζ-Potentials and diameters of the large unilamellar vesicles

Under a potential gradient of 7.2 V/cm in the particle electrophoresis apparatus, the phospholipase A_2-treated large unilamellar vesicles, which were made of a 1 : 5 molar mixture of dTPC and DSPC (Fig. 5c), migrated to the anode at a mobility (v) of 0.17 μm/s per V per cm. Under the same condition, the enzyme-untreated vesicles moved to the anode at $v = 0.09$ μm/s per V per cm. Upon setting these values to the Helmholz-Smoluchowski equation [15], $\zeta = 4\pi\eta v/\varepsilon$ ($= 12.9v$), where η and ε are the viscosity and dielectric constants of the sample solution, respectively, and they are taken as approximately equal to those of water, and the ζ-potential of the enzyme-treated and -untreated liposomes were calculated to be -2.22 mV and -1.17 mV, respectively.

Immediately after the ζ-potential measurements, the liposome solutions were subjected to light-scattering spectrometry, which gave 1930 and

Fig. 5. Electron micrographs of negatively stained liposomes with uranyl acetate. (a) 0.05 M Tris-HCl buffer solution (2.0 ml) containing a 1 : 5 molar mixture of dTPC and DSPC (total 2.7 mmol/l) was sonicated at 55 °C, at 50 W for 10 min, then incubated with phospholipase A_2 (6 μg in the 50 μl of the same buffer) at 42 °C for 15 min. (b) a 1 : 10 molar mixture of DPPC and DSPC (total 2.7 mmol/l) was transformed into liposomes and processed with the enzyme under the same conditions as those employed in a; (c) the liposomes prepared by sonicating 0.05 M Tris-HCl buffer solution (5.0 ml) containing a 1 : 5 molar mixture of dTPC and DSPC (total 0.5 mmol/l) was converted into large unilamellar vesicles by a freeze-thawing-sonicating cycle, and then treated with phospholipase A_2 (15 μg in the 125 μl of the same buffer) at 42 °C for 15 min.

1650 Å as the diameter of the enzyme-treated and -untreated vesicles, respectively (experimental error, ±5%). We noted further that the ζ-potential decreased (to a more negative value) with an increasing content of **3** (from dTPC) in the mixed liposomes and the diameter of the enzyme-treated vesicles was always somewhat larger than that of the untreated ones. Although the exact reason of the increased diameter has not been examined further, it might be ascribed to the carboxyl groups formed which extend over the liposome surface, solvating water molecules or attracting cations around them.

Discussion

Artificial dTPC resembles natural DPPC with respect to mobility and lipophilicity in its TLC and liposome-forming properties. Namely, upon sonication, both lipids furnished liposomes of 200–800 Å in diameter with similar gel-to-liquid crystalline phase transition temperature (T_c, 41–42° C); cf. Fig. 1a. The bilayer of dTPC, however, appeared not to be thermodynamically stable in comparison with that of DPPC, as judged from their phase transition enthalpies (ΔH). It is conceivable that the stability of DPPC in the bilayer can be ascribed not only to a lateral hydrophobic interaction between the long chain-hydrocarbon residues but also to a longitudinal gear-like interaction between the terminal methyl groups of the inner and outer layers of the membrane as depicted in Fig. 6. By contrast, the bilayer assembled from dTPC may lack the gear-like interaction owing to a ring structure of the hydrocarbon residue, which perhaps caused the membrane of dTPC to be thermodynamically unstable. As expected, however, sonication of dTPC in the presence of more than one equiv. of DPPC gave rise to fairly stable liposomes, as shown in Fig. 1b, showing the phase transition enthalpies between those of dTPC and DPPC.

In the second phase of the present study, we examined enzymatic susceptibilities of dTPC to phospholipase C (from *C. perfringens*) and phospholipase A_2 (from *Naja naja*) to reveal any differences between the macrocyclic lipid and DPPC. Here, phospholipase C is the enzyme that catalyzes hydrolysis of 1,2-diacyl-*sn*-glycero-3-

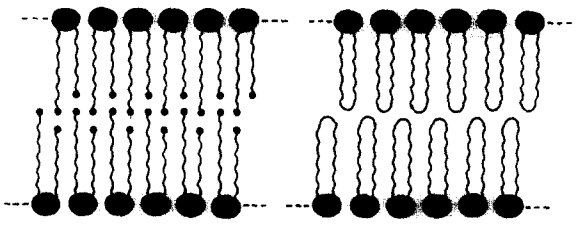
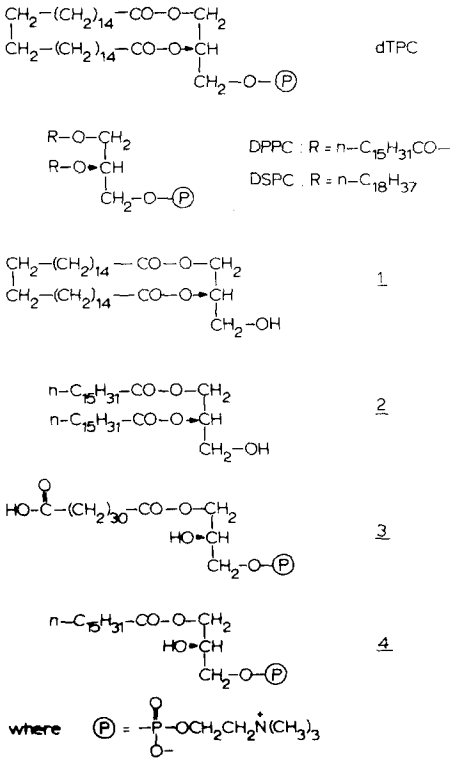


Fig. 6. Schematic representation of the membranes assembled from DPPC (left) and from dTPC (right). According to X-ray studies [16,17], the tails of the aliphatic residues of natural lipids interact each other in a gear-like fashion.

phosphocholine at the *sn*-3-phosphoester linkage [18]. Not surprisingly, dTPC was hydrolyzed readily by the enzyme to yield **1** and phosphorylcholine. However, dTPC was different from DPPC in the rate of hydrolysis (dTPC > DPPC) and in the extent of hydrolysis at the equilibrium state (dTPC > DPPC) as seen in Fig. 2. Although the mode of action of phospholipase C on glycerophospholipids has not been thoroughly



Scheme I. Structures of dTPC, DPPC, DSPC, **1**, **2**, **3**, and **4**.

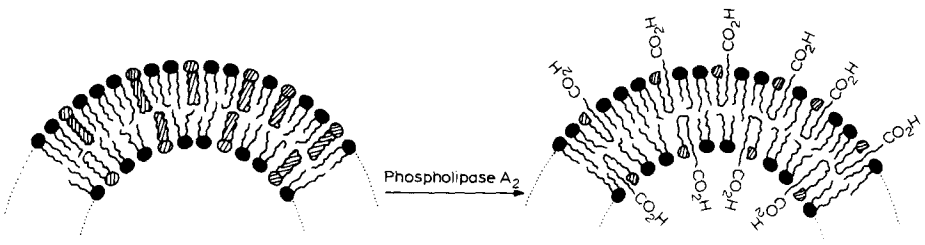


Fig. 7. Schematic representation of phospholipase A_2-catalyzed modulation of the surface of the liposomes assembled from a mixture of dTPC and DSPC. Only dTPC is hydrolyzed by the enzyme. The resulting lysolipids (**3**) are depicted in a hairpin structure; they might extend in a line to transverse the membrane.

elucidated, these results may indicate that the artificial dTPC is a better substrate for the enzyme than natural DPPC, whereas the cyclic glycerol diester (**1**) is inferior to the acyclic analog (**2**) as a substrate for the enzyme to regenerate the starting lipids. The hydrolysis of both kinds of lipid, however, took place smoothly upon addition of diethyl ether, a general activator in phospholipase-catalyzed hydrolyses, to the assay solution as shown in the broken lines in Fig. 2.

On the other hand, phospholipase A_2 is the enzyme that cleaves stereospecifically 1,2-diacyl-*sn*-glycero-3-phosphocholine at the *sn*-2-carboester linkage to yield the corresponding lysolipid and fatty acid [12]. Despite the ring structure of dTPC, the lipid was easily hydrolyzed by the enzyme to furnish the lysolipid – 1-(ω-carboxyhentriacontanoyl)-*sn*-glycero-3-phosphocholine (**3**). From quantitative enzymatic assays using the sonicated solution of DPPC alone and that of a 1 : 1 molar mixture of dTPC and DPPC, it was concluded that both kinds of lipid were very similar in the enzymatic susceptibility, and one did not affect the hydrolysis of the other in the mixed membrane (Figs. 3 and 4). The results support the previous finding that the detailed molecular structure of phospholipids is much less relevant once the minimal structural requirements are fulfilled [20].

It is remarkable that the small vesicles of a mixture of dTPC and DSPC (mole ratio, 1 : > 1) retained their structure even when all of dTPC in the liposomes was hydrolyzed into **3** upon treatment with phospholipase A_2 over the temperature range examined (25–55 °C) (Fig. 5a). By contrast, the liposomes of a mixture of DPPC and DSPC (mole ratio examined, 1 : 5 and 1 : 10) were destroyed within a few minutes, by the action of the enzyme (Fig. 5b). Apparently, unlike the lysolipid (**4**) of DPPC, **3** could work as a membrane-sustainable lysolipid. Another noteworthy consequence which has arisen from the action of phospholipase A_2 on the liposomes is an almost instantaneous generation of a new class of liposomes having CO_2H groups on the surface (Fig. 7). Formation of such liposomes was confirmed by particle electrophoresis. Namely, upon treatment with phospholipase A_2, the large unilamellar vesicles, made of a 1 : 5 molar mixture of dTPC and DSPC (Fig. 5c), which were easily observable as shiny particles under a halogen lamp, changed abruptly their ionic properties and migrated towards an anode about twice as fast as the untreated vesicles, causing the ζ-potential to decrease to -2.22 mV from -1.17 mV at 25 °C and pH 7.2 (0.05 M Tris-HCl buffer) *.

Before closing this section, we would like to describe some of the potential uses of dTPC. It is considered that the bilayer membrane made of dTPC and phospholipase A_2-resistant lipid such as DSPC would be useful as a model to study the effect of surface charge on biological transport processes and bioreactions occurring in a plasma membrane [21], because (i) dTPC is very similar to natural glycerophospholipids in its physicochemical properties (lipophilicity, surface charge and molecular dimensions). (ii) anionic charge is added easily in situ to the membrane by the action of phospholipase A_2, and (iii), as mentioned above briefly, the magnitude of the charge density or

* A ζ-potential is not influenced by the size of charged particles according to the Helmholz-Smoluchouski equation; see Results.

ζ-potential is controllable by an initially used amount of dTPC in the mixed membrane. In biological systems, phospholipase A_1 and A_2 or lysophospholipase activities might split dotriacontanedioic acid from dTPC. Their activities, however, are regulated possibly by protein kinases, transmitters, etc. [18,22], and hence the enzymes, acting as with normal plasma membrane lipids, may not work on dTPC simultaneously. From a synthetic point view of the carboxy group of **3** would be utilized to directly anchor biologically active substances on the artificial liposomes (Refs. 6 and 7; and Ref. 23 and references therein). No lipid has so far been available which has a functional carboxy group for covalent binding purposes. We are currently working in these directions.

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