Modification of Neutral (Isoelectric) Liposomes to Anionic Liposomes by Phospholipase A_2: The Use of Macrocyclic 1,2-Dotriacontanedioyl-*sn*-glycero-3-phosphocholine

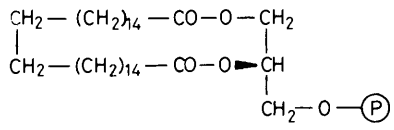
Kiyoshi Yamauchi,* Ikuo Yamamoto, and Masayoshi Kinoshita

Department of Applied Chemistry, Osaka City University, Sumiyoshi-ku, Osaka 558, Japan

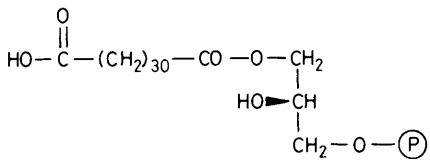
1,2-Dotriacontanedioyl-*sn*-glycero-3-phosphocholine (dTPC) has been synthesized; liposomes containing dTPC were converted rapidly into anionic liposomes at pH 7 by the action of phospholipase A_2, retaining the vesicular structure and exposing the CO_2H groups of the lysolipids on the membrane surface.

A 1:1:2 molar mixture of *sn*-glycero-3-phosphocholine, dotriacontanedioyl dichloride, and 4-*N*,*N*-dimethylaminopyridine in chloroform was stirred at 40 °C for 2 days. After silica gel, gel permeation, and high performance liquid chromatography of the reaction mixture, 1,2-dotriacontanedioyl-*sn*-glycero-3-phosphocholine (**1**), dTPC, was obtained in a yield of 8%; m.p. 240—242 °C, $[\alpha]_D^{20}$ +5.8° (*c* 0.5, $CHCl_3$).† An aqueous suspension of dTPC was then sonicated at 55 °C and 50 W. Transmission electron microscopy using a negative staining method showed the formation of small uni- and multi-lamellar vesicles (SV) having a diameter of 200—800 Å, which were similar in size and shape to the SV made of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC).

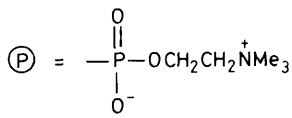
Now, we examined the substrate-specificity of dTPC to phospholipase A_2 (from *Naja naja*) (PLA_2) which catalyses the hydrolysis of *sn*-3-phospholipids at the carboester linkage in position 2.[1] Surprisingly, despite an abnormal structure of the hydrocarbon residue of dTPC, the lipid was hydrolysed smoothly by PLA_2 to afford the corresponding lysolipid—1-(ω-carboxyhentriacontanoyl)-*sn*-glycero-3-phosphocholine (**2**); m.p. 147—155 °C; $[\alpha]_D^{20}$ −2.0° [*c* 0.2, $CHCl_3$–MeOH (2:1 v/v)].† Quantitative enzymatic assays on the stable liposomes assembled from a 1:1 molar mixture of dTPC and DPPC (each 0.7 mM l^{-1}) at pH 7.2 [0.05 M $(HOCH_2)_3CNH_2$ (tris)–HCl] revealed that (i) both kinds of lipids had an optimal temperature of about 40 °C, which agreed with the gel-to-liquid crystalline phase transition temperatures measured by differential scanning calorimetry (dTPC, 42.6 °C; DPPC, 41.2 °C), with essentially similar extents of hydrolysis at 42 °C: about 80% (3 min) and about 95% (12 min). (ii) At lower (32 and 37 °C) and higher (47 and 52 °C) temperatures dTPC was processed by PLA_2 even faster than DPPC, though the hydrolysis rates of both lipids were diminished from the values observed at 42 °C.‡



(**1**) (dTPC)



(**2**)



† Compounds (**1**) and (**2**) had i.r., n.m.r., and fast atom bombardment mass spectra which agreed with the assigned structures.

‡ Wilschut and coworkers reported an asymmetric degradation of only one side of the membrane composed of a 1:1 molar mixture of egg yolk phosphatidylcholine and phosphatidylethanolamine by PLA_2 (bee venom); J. C. Wilschut, J. Regts, and G. Scherphof, *FEBS Lett.*, 1979, **98**, 181. We did not observe the 50% hydrolysis upon treatment of the liposomes made of DPPC alone or the 1:1 molar mixture of dTPC and DPPC with PLA_2 (*Naja naja*). The discrepancy between our results and those of the Wilschut group is unexplained at present. However, we did observe nearly 50% hydrolysis by treating liposomes made of DTPC or DPPC, when the inner and outer domains of the liposomes were adjusted to pH 7 and 3, respectively, then PLA_2 (*Naja naja*) was allowed to act on the liposome solution.

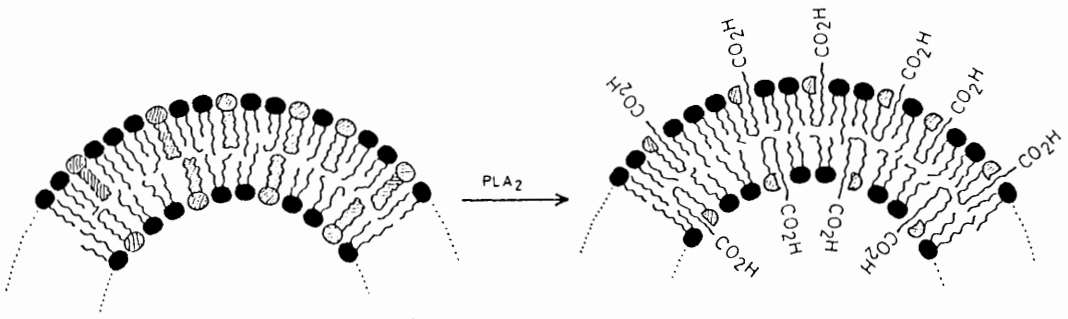


Figure 1. Schematic representation of PLA_2-catalysed modification of liposomes made from dTPC and DSPC. The lysolipids (**2**) are depicted in a hairpin structure; they might extend in a line to transverse the membrane. DSPC is not a substrate of the enzyme.

Transmission electron microscopy indicated that liposomes made from a mixture of dTPC and PLA_2-unreactive 1,2-di-stearyl-*sn*-glycero-3-phosphocholine (DSPC) (molar ratio 1 : >1) always retained their vesicular structure even when all dTPC molecules were hydrolysed to (**2**) upon treatment with PLA_2 at 25—55 °C. By contrast, liposomes of a mixture of DPPC and DSPC (molar ratios examined 1 : 5 and 1 : 10) were destroyed within a few minutes by the action of PLA_2. Apparently, unlike 1-palmitoyl-*sn*-glycero-3-phosphocholine derived from DPPC, compound (**2**) could work as a membrane-sustainable lysolipid. Another noteworthy consequence of the action of PLA_2 on the liposomes is the almost instantaneous generation of a new class of liposomes having CO_2H groups on the surface (Figure 1). The formation of such liposomes was confirmed by particle electrophoresis. Namely, addition of PLA_2 to a solution of large unilamellar vesicles (LUV) of diameter *ca.* 2000 Å at 42 °C, which was prepared by freeze–thawing the SV solution of a 1 : 5 molar mixture of dTPC and DSPC in 0.05 M tris-HCl buffer of pH 7.2, caused the ionic character of the vesicles to change abruptly, and the shining particles observed under a halogen lamp migrated toward an anode about twice as fast as the PLA_2-untreated ones. From the mobilities, the ξ-potentials of the PLA_2 -treated and -untreated liposomes were calculated to be −2.22 and −1.17 mV, respectively, at 25 °C. We noted further that the ξ-potential decreased (to more negative values) with increasing content of dTPC in the mixed liposomes while the size of the PLA_2-treated LUVs remained similar to that of the untreated ones; for instance, dynamic light scattering spectrometry of the aforementioned liposome solutions at 25 °C gave 1930 and 1650 (±5%) Å as a weight-average diameter of the PLA_2-treated and -untreated liposomes, respectively.

These observations suggest that dTPC and analogous macrocyclic phospholipids may be used in place of DPPC and other natural lipids, providing novel liposomes which can be transformed into anionic ones *in situ* by cellular lipases such as PLA_2. To date, anionic liposomes for therapeutic purposes have been obtained only by mixing anionic amphiphiles such as phosphatidic acid and oleic acid with basal lipids such as DPPC.[2—5] From a synthetic view of point, the carboxy groups of (**2**) may be utilized to anchor biologically active substances to the liposomes.

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