Alkyl Chain Length Dependency in Hydrolysis of Liposomal Phosphatidylcholine by Dialkylphosphate

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Because an amphiphile with a positive or negative charge, such as dialkylphosphate or stearylamine, is often added to liposomal phosphatidylcholine (PC) dispersions to prevent aggregation of the liposomes, we investigated the long-term stability of liposomes prepared from saturated PC in the presence of various amphiphiles. On storage of these liposomes at 40°C, PC was gradually hydrolyzed by dialkylphosphate, a negatively charged lipid, while neither stearylamine, a positively charged lipid, nor a non-charged lipid hydrolyzed PC at all. This hydrolysis of PC was examined using dialkylphosphates of various alkyl chain lengths (C10, C12, C14, C16, C18 and C20) and PC with different fatty acyl chain lengths (C14, C16 and C18). The rate of hydrolysis was maximum when the alkyl chain length of dialkylphosphate was almost equal to the fatty acyl chain length of PC. That is, the hydrolysis of dimyristoyl (C14) and dipalmitoyl (C16) acyl chains of PC was accelerated most by the incorporation of dimyristylphosphate (C14) and dipalmitylphosphate (C16), respectively. Distearoyl (C18) acyl chains of PC were hydrolyzed effectively by the incorporation of distearylphosphate (C18) as well as dipalmitylphosphate (C16). The hydrolysis did not occur when methyl dipalmitylphosphate was added instead of dipalmitylphosphate, or when the liposomal structure was decomposed by adding ethanol. These results suggest that dialkylphosphate and PC are aligned head to tail in liposomes and that the phosphate functional group causes the hydrolysis of the esters of PC.

The incorporation of cholesterol into PC bilayers suppressed the hydrolysis of PC above the phase transition temperature (T_c) of PC, but increased it below the T_c. The hydrolysis of PC by dialkylphosphate appears to depend on membrane fluidity and to be accelerated with increased membrane fluidity, because cholesterol reduces the fluidity of the liposomal membrane above the T_c and enhances it below the T_c.

Key words hydrolysis; liposome; dialkylphosphate; phosphatidylcholine; decomposition

Though some liposomal products are on the market as pharmaceuticals, the stability of liposomes during long-term storage for practical use still depends on their physical and chemical properties.[1] We have reported the oxidative decomposition of liposomes composed of unsaturated phosphatidylcholine (PC), followed by a decrease in pH, during long-term storage. Further, liposomes composed of saturated PC are hydrolyzed during storage, though the mechanism has not been established.

An amphiphile with a positive or negative charge is often added to liposomal dispersions for practical use, to prevent aggregation.[2] We found that dialkyl phosphate, a lipid with a negative charge, causes the hydrolysis of liposomal PC, and so we examined the hydrolysis in detail by using dialkylphosphates of various alkyl chain lengths (C10, C12, C14, C16, C18 and C20) and PCs with three different fatty acyl chain lengths (C14, C16 and C18). The results are presented here.

Results

Dialkylphosphate-Induced Hydrolysis of Liposomal PC Lipid decomposition was monitored by measuring the amounts of PC, glycerophosphorylcholine (GPC) and fatty acids (FA) in the liposomal suspension. When saturated PC liposomes incorporating dipalmitylphosphate were incubated at 40 °C, insoluble precipitates composed of fatty acids appeared. Table 1 shows the effect of amphiphiles on the change in PC content in hydrogenated soya phosphatidylcholine (H-Soya PC) liposomes after 28 d at 40 °C. Stearylamine, a positively charged lipid, did not influence PC stability, but dipalmitylphosphate, a negatively charged lipid, significantly decomposed PC. The results for H-Soya PC liposomes incorporating dipalmitylphosphate are summarized in Table 2. The decrease in PC content and corresponding increases in GPC and FA were observed during storage. GPC increased with the decrease of PC at a 1 : 1 ratio, while FA increased at a

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Table 1. Effect of Charged Amphiphiles on Decomposition of PC in H-Soya PC Liposomes

Treatment	Residual PC (%)
No addition	96
Stearylamine (1 mM)	97
Dipalmitylphosphate (1 mM)	77

H-Soya PC liposomes incorporating stearylamine (1 mM) or dipalmitylphosphate (1 mM) were incubated for 28 d at 40°C. The initial pH of liposomes was adjusted to 7.50 ± 0.05 by the addition of 0.1 N NaOH. The initial value of H-Soya PC was 10 mM.

Table 2. Decomposition of H-Soya PC Liposomes

	0 d	7 d	14 d	28 d
PC (mM)	9.5	8.5	7.9	6.6
GPC (mM)	0.1	1.2	1.7	2.9
FA (mM)	0.2	2.5	3.4	5.8

H-Soya PC liposomes incorporating dipalmitylphosphate (1 mM) were incubated for 28 d at 40 °C. The initial pH of liposomes was adjusted to 7.50 ± 0.05 by the addition of 0.1 N NaOH. The initial concentration of H-Soya PC was 10 mM.

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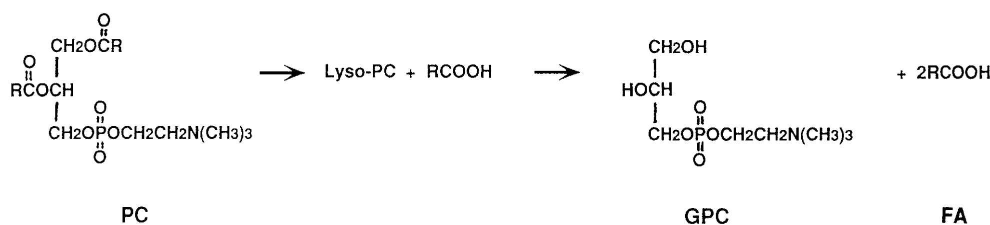

Fig. 1. Hydrolysis Reaction of Phosphatidylcholine (PC)

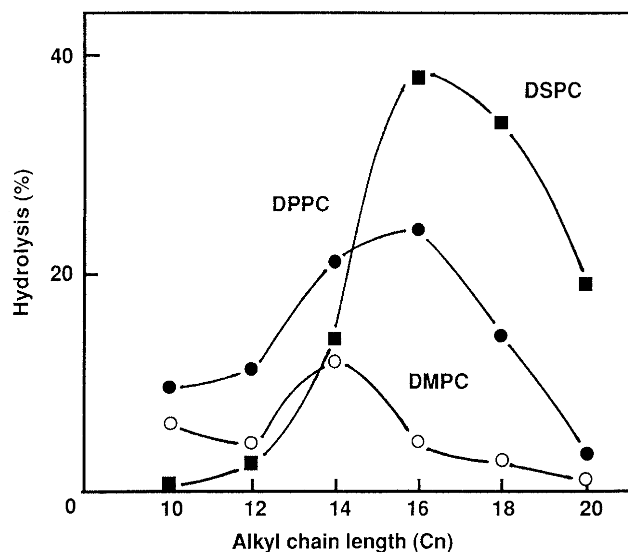

Fig. 2. Effect of Alkyl Length of Dialkylphosphate on Hydrolysis of PC

Liposomes incorporating dialkylphosphate (2 mM) were incubated for 7 d at 40 °C. The initial pH of liposomes was adjusted to 7.00±0.05 by the addition of 0.1 N NaOH. The initial concentration of DMPC, DPPC or DSPC was 10 mM.

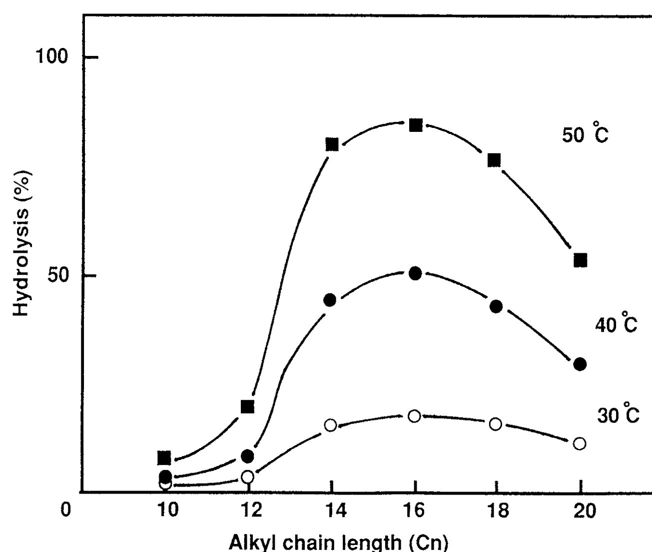

Fig. 3. Effect of Temperature on Hydrolysis of DPPC Liposomes

DPPC liposomes incorporating dialkylphosphate (2 mM) were incubated for 7 d at 40 °C. The initial pH of liposomes was adjusted to 7.00±0.05 by the addition of 0.1 N NaOH. The initial concentration of DPPC was 10 mM.

2:1 ratio. This result indicates that PC was completely hydrolyzed into GPC and FA (Fig. 1).

Phosphate–choline binding was not cleaved under these conditions because the amount of phospholipid phosphorus in the water layer after extraction of the liposomal dispersion with chloroform–methanol was equal to the amount of phosphorus of GPC. No phosphatidic acid was generated in the hydrolysis reaction. So, GPC is the only water-soluble decomposition product with a phospholipid phosphorus structure.

Effect of Alkyl Chain Length of Dialkylphosphate on Hydrolysis of PC Liposomes incorporating dialkylphosphate of various alkyl chain lengths were incubated at 40 °C, and the amount of GPC formed was examined. Figure 2 shows the effect of alkyl chain length of dialkylphosphate on the hydrolysis of PC. In L-α-dimyristoyl-phosphatidylcholines (DMPC: C14) and L-α-dipalmitoyl-phosphatidylcholines (DPPC: C16) liposomes, the hydrolysis of PC was most enhanced by the incorporation of dimyristylphosphate (C14) and dipalmitylphosphate (C16), respectively. When L-α-distearoyl-phosphatidylcholines (DSPC: C18) liposomes were used, PC was hydrolyzed effectively by the incorporation of dipalmitylphosphate (C16) or distearylphosphate (C18). These results indicate that the hydrolysis of PC was most accelerated when the alkyl chain length of dialkylphosphate was almost equal to the fatty acyl chain length of PC, and was greatly decreased when the chain length differed by 2 or 4 carbons. The maximum rate of hydrolysis increased with an increase in the fatty acyl chain length of PC.

Effect of Temperature on Hydrolysis of PC DPPC liposomes incorporating dialkylphosphate of various alkyl chain lengths were incubated at various temperatures. As shown in Fig. 3, the rate of hydrolysis of PC increased with an increase in the incubation temperature. This result indicates that the hydrolysis of PC by dialkylphosphate is greatly affected by liposomal membrane fluidity. The rate of hydrolysis was highest when dipalmitylphosphate was incorporated into the liposomal membrane, independent of the storage temperature, suggesting that the acyl chain length of liposomes composed of saturated PC had a similar effect on the hydrolysis by dialkylphosphate within the storage temperature range of 30—50 °C.

Effect of Dialkylphosphate Concentration and Cholesterol Incorporated into Membranes on Hydrolysis of Liposomes Figure 4 shows the effect of dipalmitylphosphate concentration and cholesterol incorporation on the hydrolysis of DPPC liposomes. The hydrolysis of DPPC was accelerated with an increase in dipalmitylphosphate concentration at any temperature. This observation strongly supports the view that the hydrolysis of DPPC liposomes is caused by dipalmitylphosphate. When cholesterol at 75 mol percent of PC was incorporated into liposomes, the hydrolysis was suppressed at 50 °C (above the T_c of DPPC) but increased at 30 °C (below the T_c).

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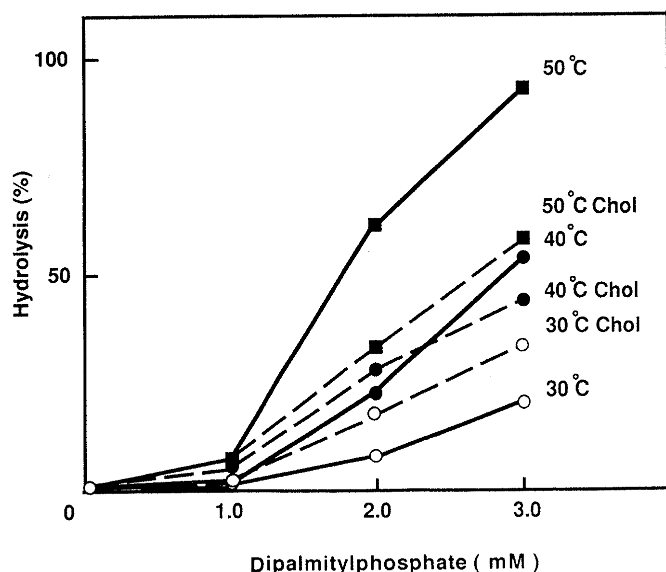

Fig. 4. Effect of Dipalmitylphosphate Concentration and Cholesterol Incorporation on Hydrolysis of DPPC Liposomes

DPPC liposomes incorporating cholesterol (molar ratio cholesterol/DPPC = 0.75) and dipalmitylphosphate were incubated for 7 d at 40 °C. The initial pH of liposomes was adjusted to 7.00 ± 0.05 by the addition of 0.1 N NaOH. The initial concentration of DPPC was 15 mM.

These results indicate that the hydrolysis of PC induced by dialkylphosphate depends on membrane fluidity, and is accelerated with increased membrane fluidity, since cholesterol reduces the fluidity of the liposomal membrane above the T_c, but enhances it below the T_c.

Discussion

For the practical application of liposomes, long-term stability over a wide range of temperature is very important. The hydrolysis of PC has been investigated as a function of pH, temperature, buffer concentration and buffer species.[3] But little has been reported about the chemical stability of PC in the presence of amphiphiles. In this work, we investigated the long-term stability of liposomes prepared from saturated PC in the presence of negatively charged lipids. It is known that saturated PC has maximum stability at a neutral pH.[4] However, we have found that saturated PC is drastically hydrolyzed in the presence of dialkylphosphate, even at a neutral pH. In the absence of dipalmitylphosphate or in the presence of stearylamine, a positively charged lipid, no hydrolysis of PC occurred, suggesting that this hydrolysis depends on the dialkylphosphate. Negatively charged lipids, especially dipalmitylphosphate, have commonly been used for preventing the aggregation of liposomes, but it is now clear that the hydrolysis of PC induced by dipalmitylphosphate will cause a breakdown of liposomal structure and subsequent release of the contents. Therefore we suggest that the incorporation of negatively charged lipids, especially those with the same alkyl chain length as the acyl chain length of PC, should be avoided to ensure the long-term stability of liposomes. The hydrolysis of PC by dialkylphosphate was not observed when the liposomal structure was decomposed by the addition of ethanol before storage, and no hydrolysis of PC occurred when methyl dipalmitylphosphate was used instead of dipalmitylphosphate. These results may indicate that the hydrolysis of PC by dialkylphosphate is sensitive to phospholipid packing, especially the relative position of PC and the head group of dialkylphosphate. Kensil and Dennis[5] investigated alkaline hydrolysis rates and activation energies for hydroxide ion attack on PC in various aggregation states (mixed micelles, multibilayers, vesicles and monomers) and found them to depend critically on the lipid aggregation state. They showed that egg PC dispersed in single bilayer vesicles and multibilayers was much less hydrolyzed than PC in Triton X-100 micelles or PC monomers. These results are quite different from those on the hydrolysis of PC by dialkylphosphate, suggesting that the hydrolysis of PC by dialkylphosphate in liposomes requires the liposomal structure of phospholipid.

In this study, we have shown that the hydrolysis of liposomal PC occurs to the greatest extent when the alkyl chain length of dialkylphosphate is almost equal to the fatty acyl chain length of PC, and is greatly decreased when the chain lengths differ by 2 or 4 carbons. The position of the headgroup of dialkylphosphate in relation to the ester bond of PC may be very important for the rate of hydrolysis. Two lipids which differ by only 2 or 4 carbons in the lengths of their hydrocarbon chains appear to form a nearly ideal mixture,[6] indicating that the great decrease in hydrolysis when the chain lengths differ by 2 or 4 carbons is not due to the phase separation of PC. These results suggest that dialkylphosphate and PC are aligned strictly head to tail in liposomes and that the phosphate functional group causes the hydrolysis of the ester moiety in PC.

The rate of hydrolysis was temperature-dependent. Cholesterol incorporation into the liposomal membranes suppressed the hydrolysis rate above the T_c but increased it below the T_c. Cholesterol is often incorporated into liposomal membranes to stabilize their structure.[7] It has been suggested that cholesterol has the function of controlling the fluidity of hydrocarbon chains in the lipid bilayer of membranes. These findings may indicate that the rate of hydrolysis of PC caused by dialkylphosphate is influenced by the membrane fluidity or the rate of lateral diffusion of the substrate on the membrane.

Conclusions

We investigated the long-term stability of liposomes prepared from saturated PC in the presence of negatively charged lipids. On storage of these liposomes at 40 °C, we have found that saturated PC is drastically hydrolyzed in the presence of dialkylphosphate, even at a neutral pH. In the absence of dialkylphosphate or in the presence of stearylamine, a positively charged lipid, no hydrolysis of PC occurred, suggesting that this hydrolysis depends on the dialkylphosphate.

The rate of hydrolysis was maximum when the alkyl chain length of dialkylphosphate was almost equal to the fatty acyl chain length of PC. The hydrolysis of PC by dialkylphosphate was sensitive to phospholipid packing, especially the relative position of PC and the head group of dialkylphosphate.

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incorporation of cholesterol into PC bilayers suppressed the hydrolysis of PC above the T_c of PC but increased it below the T_c. The hydrolysis of PC by dialkylphosphate appears to be influenced by membrane fluidity and to be accelerated with increased membrane fluidity, since cholesterol reduces the fluidity of the liposomal membrane above the T_c, but enhances it below the T_c.

Experimental

Materials H-Soya PC (95% purity), DMPC, DPPC and DSPC (99% purity) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Dipalmitylphosphate, peroxidase and GPC phosphodiesterase were obtained from Sigma Chemical Co. Stearylamine, 4-aminoantipyrine and phenol were from Tokyo Kasei Kogyo, Ltd. Choline oxidase was from Funakoshi Co., Ltd. All other chemicals were commercial products of reagent grade.

Preparation of Liposomes Multilamellar vesicles were prepared by the method of Bangham *et al.*[2a] The lipids were dissolved in chloroform–methanol (2:1, v/v) in a small round-bottomed flask and then the chloroform–methanol was evaporated off under a vacuum to obtain the dried lipid film, which was hydrated with an aqueous solution containing 0.1% *p*-hydroxybenzoic acid methyl ester. The flask was agitated on a Vortex mixer for more than 5 min until the lipid film had been freed from the bottom of the flask. The temperature was maintained above the gel-liquid crystalline phase transition temperature (T_c) of the lipid materials. The pH of the liposomal suspension was adjusted to 7.00 or 7.50±0.05 by the addition of 0.1 N NaOH.

Lipid Analysis The amount of PC was determined by enzymatic assay using a Phospholipids C-test Wako (Wako Pure Chemical Industries, Ltd.). The amount of GPC was determined in terms of the formation of quinoneimine dye according to the method of Tokura *et al.*[8] In brief, the reaction system (the total volume was 3 ml) contained 0.4 U of GPC phosphodiesterase, 5 U of choline oxidase, 1 mM $CaCl_2$ and the peroxidase system. The peroxidase system (2.0 ml) contained 5.0 U of peroxidase, 2.25 mM 4-aminoantipyrine, 3.15 mM phenol, 0.5% Triton X-100 and 0.1 M Tris–HCl (pH 8.0). To this reaction system, 0.1 ml of liposomal dispersion, diluted appropriately, was added and the whole was incubated at 37 °C for 15 min. Hydrogen peroxide produced from choline by choline oxidase was assayed in terms of the formation of quinoneimine dye by peroxidase. The formation of quinoneimine dye was monitored at 500 nm. A mixture containing choline chloride in place of the liposomal dispersion was used as a standard.

Other Analytical Methods The quantity of FA formed as hydrolysis products was determined by gas-liquid chromatography. The fatty acids were analyzed on a fused silica megabore column (0.53 mm × 15 m) coated with DB-1 (J & W Co.) from 60 °C to 250 °C (10 °C/min). The flow rate of helium was 52.5 ml/min. Phospholipid phosphorus was determined by the method of Hoeflmayr and Fried[9] using a Phospholipids-test Wako.

Syntheses of Dialkylphosphates All chemicals were of reagent grade, from Sigma Chemical Co. and Tokyo Kasei Kogyo, Ltd. P_2O_5 was added dropwise to alkylalcohol and the reaction mixture was heated at 80 °C for about 24 h with stirring. After being cooled to room temperature, the reaction mixture was filtered with suction. The residue was thoroughly washed with five 100 ml portions of water, and recrystallized from ethyl acetate.

C10; *Anal.* Calcd for $C_{20}H_{43}O_4P$: C, 63.46; H, 11.44. Found: C, 63.15; H, 11.71. mp 44 °C.

C12; *Anal.* Calcd for $C_{24}H_{51}O_4P$: C, 66.32; H, 11.82. Found: C, 66.03; H, 12.12. mp 57 °C.

C14; *Anal.* Calcd for $C_{28}H_{59}O_4P$: C, 68.53; H, 12.11. Found: C, 68.24; H, 12.36. mp 66 °C.

C16; *Anal.* Calcd for $C_{32}H_{67}O_4P$: C, 70.22; H, 12.42. Found: C, 69.92; H, 12.71. mp 70 °C.

C18: *Anal.* Calcd for $C_{36}H_{75}O_4P$: C, 71.71; H, 12.53. Found: C, 71.42; H, 12.80. mp 76 °C.

C20: *Anal.* Calcd for $C_{40}H_{83}O_4P$: C, 72.89; H, 12.69. Found: C, 72.62; H, 12.90. mp 85 °C.

Synthesis of Methyldipalmitylphosphate To a dispersion of dipalmitylphosphate in 150 ml of chloroform, a solution of diazomethane in dry diethyl ether was added from a buret at a rate of about 2 ml per minute with stirring at 0 °C, until the yellow color of diazomethane persisted for several minutes. Then the reaction mixture was left standing at room temperature for several minutes, filtered to remove the residual dipalmitylphosphate and evaporated to dryness. The residue was dried under a vacuum to afford a white powder, which was recrystallized from cold methanol.

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