pH Lowering in Liposomal Dispersions Induced by Phospholipid Peroxidation

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Liposomes were prepared from egg phosphatidylcholine (PC) for investigation of their long-term stability. When egg PC liposomes were incubated at 40 °C, lowering of the pH was observed with a corresponding change in appearance. This pH lowering was suggested to be induced by peroxidation of the unsaturated fatty acyl chains in egg PC because: 1) the pH lowering was prevented by incorporation of α-tocopherol into liposomes or bubbling nitrogen into the liposomal dispersion at neutral pH; 2) the liposomes prepared from completely hydrogenated soya PC (CH-Soya PC) showed no change in pH under the same conditions. The relationship between the pH lowering and lipid peroxidation was also supported by the formation of thiobarbituric acid-reactive substances (TBA-RS) and the accumulation of carbonyl compounds in liposomes. The amount of TBA-RS reached a maximum after about 5 days' storage and then decreased gradually, while the pH lowering and production of carbonyl compounds in the liposomal dispersion continued for 20 d. These results indicate that production of TBA-RS does not directly correlate with pH lowering in the liposomal dispersion; rather TBA-RS might be decomposed into secondary peroxidation products, such as carbonyl compounds, which may affect the pH of the liposomal dispersion.

Key words liposome; peroxidation; pH lowering; phospholipid; phosphatidylcholine; carbonyl compound

Liposomes consisting of phospholipids have been widely used as a tool for studying the structure and function of living cell membranes.[1] The liposomal bilayer itself can store amphiphilic and lipophilic active ingredients and these characteristics have led to various biological applications, such as in drug delivery systems.[2] It has been recognized that liposomes have great potential as drug carriers in medical applications since they can reduce drug toxicity and target drugs to specific cells and organs.[3] Liposomes are also effective for dermato-pharmacotherapy.[4]

However, the instability of liposomes presents a serious obstacle to their large-scale medical use, because of decomposition during storage under various conditions. Many investigations have been come out to resolve this instability problem. Kikuchi *et al.*[5] have recently reported that liposomes can be kept stable during long-term storage (*e.g.*, 6 months at 25 °C) by bubbling nitrogen into the liposomal solution, but this may not be a practical approach. A change in pH should be an important factor influencing the physicochemical stability of liposomes, and so it is very important to investigate the pH change of liposomal dispersions during storage. In this work, we examined the cause of the pH lowering in a stored liposomal dispersion.

Experimental

Materials Egg PC with an iodine value of 66 g/100 g and a peroxide value (*POV*) of 1.6 meq/kg and completely hydrogenated soya PC with an iodine value of 0.1 g/100 g and a *POV* of 0.6 meq/kg (CH-Soya PC) were obtained from Nippon Oils and Fats Co., Ltd. (Tokyo). Dicetylphosphate (DCP) was from Sigma Chemical Co. and α-tocopherol, 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid, 2,4-dinitrophenylhydrazine and $FeCl_2 \cdot 4H_2O$ were obtained from Wako Pure Chemical Industries Ltd. 3-*tert*-Butyl-4-hydroxyanisole (BHA) was from Tokyo Kasei Kogyo Ltd. and all other chemicals were commercial products of reagent grade.

Preparation of Liposomes Multilamellar vesicles were prepared by the method of Kikuchi and Yamauchi.[6] Lipids were added to glycerol which was heated above the phase transition temperature (T_c) of the liquid materials. The mixture was hydrated with 200 ml aqueous 0.1% *p*-hydroxybenzoic acid methyl ester preheated to above the T_c and allowed to stand for 3 min. The phospholipid and DCP concentrations were adjusted to 10 and 1 mM, respectively. The lipid dispersion was agitated by mixing at 6000 rpm for 3 min above the T_c of the lipid materials. The pH of the liposomal dispersion was adjusted to 7.60 ± 0.05 by adding 1 N NaOH. The mean liposome particle size was determined by quasi-elastic laser light scattering (QELS) measurements using a Coulter model N4SD (Coulter Electronics, Inc.). The mean vesicle size of the liposomes was about 200 nm.

Measurement of Lipid Peroxidation The amount of thiobarbituric acid-reactive substances (TBA-RS) was estimated by the method of Buege and Aust[7] and was expressed as equivalents of malondialdehyde (the hydrolysate of 1,1,3,3-tetraethoxypropane). Carbonyl compounds were assayed by the formation of 2,4-dinitrophenylhydrazone derivatives according to the method of Lappin and Clark.[8] An equal volume of 0.05% 2,4-dinitrophenylhydrazine in methanol and one drop of concentrated HCl were added to 1 ml of liposomal dispersion after appropriate dilution. After incubation at 50 °C for 30 min, 5 ml 10% HCl was added to the incubation mixture. The mixture was filtered through a 0.45 μm membrane filter, and the absorbance measured at 480 nm. To determine the distribution of carbonyl compounds in liposomes, 5 vol. chloroform–methanol (1 : 1) was added to 1 vol. liposomal dispersion. Non-polar and hydrophilic compounds were extracted into the chloroform and water layers, respectively. The amount of carbonyl compounds in each layer was determined as described above.

Lipid Analysis The amount of PC was determined by enzymatic assay using a Phospholipids C-test Wako kit (Wako Pure Chemical Industries Ltd.). The fatty acid composition of the 2 types of PC was determined by gas-liquid chromatography. Phospholipids were dissolved in 20 ml 3.5% w/v KOH ethanol–water (100 : 2) and hydrolyzed at 80 °C. The mixture was cooled and the ethanol was evaporated under reduced pressure. Twenty ml water was added to the residue and the mixture was acidified with 6 N HCl and extracted three times with 50 ml portions of diethyl ether. The extracts were combined, washed three times with 20 ml portions of water and evaporated. The residue was methylated with *N*,*N*-dimethylformamide (DMF)–dimethyl acetal and the methyl esters were analyzed on a column (3 mm × 2 m) of 5% Silar 10C on Chromosorb WAW DMCS (Gasukuro Kogyo Co.).

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Results

pH Lowering in Egg PC Liposomal Dispersions When the liposomal dispersion composed of egg PC was incubated for 2 weeks at 40 °C, the pH of the liposomal dispersion fell gradually to 6.5 (Fig. 1). This pH change was not observed when CH-Soya PC was used instead of egg PC. Egg PC contains both unsaturated and saturated fatty acids, whereas CH-Soya PC contains exclusively saturated fatty acids (Table 1). These results suggest that the pH lowering is due to the unsaturated fatty acyl chains. As shown in Table 2, the addition of 20 μM $FeCl_2$ to the dispersion of egg PC liposomes accelerated the pH lowering. Bubbling nitrogen through the liposomal dispersion before incubation suppressed the pH lowering and incorporation of BHA or α-tocopherol into the egg PC liposomes also prevented it. Concomitant α-tocopherol incorporation and nitrogen bubbling was even more effective. These results suggest that peroxidation of unsaturated fatty acyl chains is involved in the pH lowering of liposomal dispersions.

Formation of Carbonyl Compounds in Liposomal Dispersions Tables 3 and 4 demonstrate the relationship between the amount of carbonyl compounds and the pH in egg PC liposomal dispersions after 2 weeks. The pH of the liposomal dispersion fell as the concentration of carbonyl compounds increased with increasing incubation temperature (Table 3).

Formation of carbonyl compounds in egg PC liposomal dispersions was examined under various conditions (Table 4). Under atmospheric oxygen, the amount of carbonyl compounds in egg PC liposomal dispersions was higher than that observed in the presence of nitrogen and the incorporation of BHA suppressed both the formation of carbonyl compounds and the pH lowering in egg PC liposomal dispersions. The addition of 20 μM $FeCl_2$ to dispersions of egg PC liposomes enhanced the increase in carbonyl compounds and pH lowering. These results show that accumulation of carbonyl compounds is responsible for the pH lowering in liposomal dispersions incubated for a long period.

Time-Courses of pH Change, Carbonyl Compound Production, and TBA-RS Production When egg PC liposomal dispersions were incubated at 30 °C, the amount of TBA-RS increased almost linearly, to reach a maximum within 5 d and thereafter declined gradually. At higher temperature (50 °C), the amount of TBA-RS increased, but then declined quickly after 5 d (Fig. 2c). In addition, pH lowering and production of carbonyl compounds in the liposomal dispersions occurred simultaneously up to 15 d (Figs. 2a and 2b). These results suggest that production of TBA-RS is not directly correlated with pH lowering; instead, TBA-RS may be decomposed into secondary peroxidation products such as carbonyl compounds, which would affect the pH of liposomal dispersions.

Distribution of Carbonyl Compounds Carbonyl compounds in the liposomal dispersions were extracted with

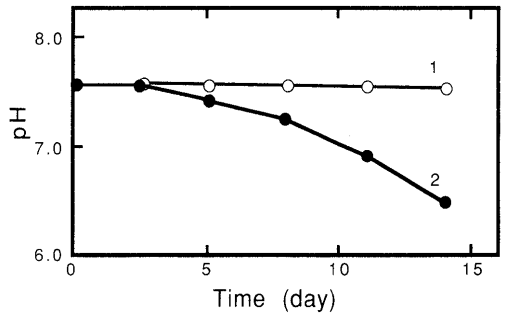

Fig. 1. pH Change in an Egg PC Liposomal Dispersion during Storage

The plots show the change in pH of egg PC and CH-Soya PC liposomal dispersions during incubation for 2 weeks at 40 °C in air. Line 1, CH-Soya PC; line 2, egg PC.

Table 1. Fatty Acid Compositions of Egg PC and CH-Soya PC

Lipid	Fatty acid composition (%)					
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	$C_{22:6}$
Egg PC	32.5	13.5	29.2	13.6	3.0	4.4
CH-Soya PC	11.0	88.0	0	0	0	0

Egg PC and CH-Soya PC were hydrolyzed and esterified as described in the experimental section. Values are expressed as mol% total fatty acids.

Table 2. Effects of Ferrous Chloride ($FeCl_2$) and Antioxidants on the pH Change of Egg PC Liposomal Dispersions

Treatment	pH after storage
No addition	6.45
$FeCl_2$ (20 μM)	6.18
N_2 gas	7.30
BHA (2 mM)	7.23
VE (2 mM)	7.38
VE (2 mM), N_2 gas	7.72

Egg PC liposomal dispersions were incubated for 2 weeks at 40 °C after the indicated treatments. The initial pH of the liposomal dispersions was adjusted to 7.60±0.05 by adding 1 N NaOH.

Table 3. Relationship between the Amount of Carbonyl Compounds and pH

Incubation temperature (°C)	Carbonyl compounds (μmol/μmol PC)	pH
30	0.6250	6.96
40	0.7132	6.72
50	0.8194	6.35

Egg PC liposomal dispersions were incubated for 2 weeks at the indicated temperature in air. The initial pH of the liposomal dispersions was adjusted to 7.60±0.05 by adding 1 N NaOH. The amount of carbonyl compounds is expressed as μmol acetaldehyde per μmol PC.

Table 4. Relationship between the Amount of Carbonyl Compounds and pH under the Various Conditions

Treatment	Carbonyl compounds (μmol/μmol PC)	pH
No addition	0.7132	6.72
N_2 gas	0.0217	7.42
BHA (2 mM)	0.2534	7.26
$FeCl_2$ (20 μM)	0.8352	6.42

Egg PC liposomal dispersions were incubated for 2 weeks at 40 °C after the indicated treatments. The initial pH of liposomal dispersions was adjusted to 7.60±0.05 by adding 1 N NaOH. The amount of carbonyl compounds is expressed as μmol acetaldehyde per μmol PC.

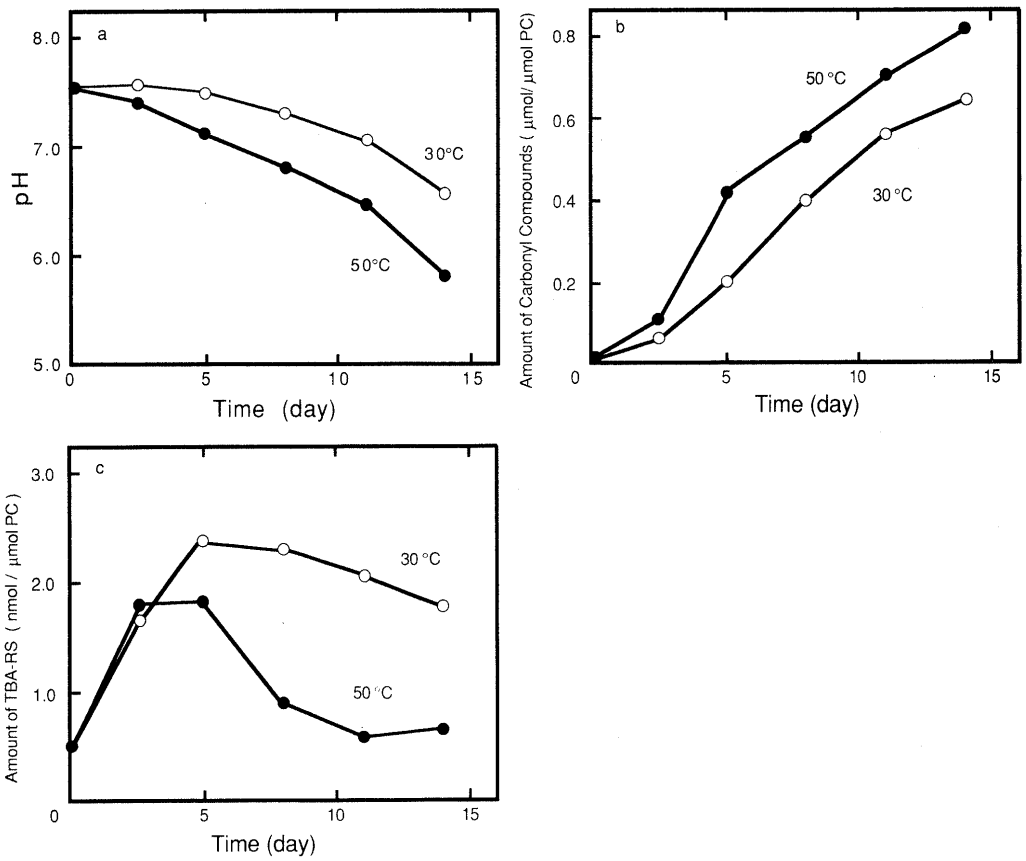

Fig. 2. Time–Courses of pH Change (a), Carbonyl Compound Production (b), and TBA-RS Production (c)

Egg PC liposomal dispersions were incubated for 2 weeks at 30 °C or 50 °C in air. The amount of TBA-RS is expressed as nmol malondialdehyde per μmol PC. The amount of carbonyl compounds is expressed as μmol acetaldehyde per μmol PC.

Table 5. Distribution of Carbonyl Compounds in Liposomal Dispersions

Treatment	Carbonyl compounds (μmol/μmol PC)	
	Organic layer	Water layer
No addition	0.5463	0.1670
N_2 gas	0.0170	0.0051
$FeCl_2$ (20 μM)	0.6258	0.2183

Egg PC liposomal dispersions were incubated for 2 weeks at 40°C after the indicated treatments, and then the distribution of carbonyl compounds in the dispersions was examined by extraction with chloroform–methanol (1 : 1) after incubation. The amount of carbonyl compounds in each layer was determined as described in the experimental section, and is expressed as μmol acetaldehyde per μmol PC.

Table 6. Change in Liposomal Phospholipids after Storage

Treatment	PC (μmol/ml)
No addition	9.54
N_2 gas	9.96

Egg PC liposomal dispersions were incubated for 2 weeks at 40 °C in air or nitrogen. The initial value of PC was 10 μmol/ml. One ml of liposomal dispersion was diluted with ethanol to 10 ml, then the amount of PC was measured by enzymatic assay.

chloroform–methanol (1 : 1) after incubation.

As shown in Table 5, carbonyl compounds were detected in both organic and aqueous layers, but the amount of carbonyl compounds in the chloroform–methanol layer was higher, suggesting that many phosphatidylcholine derivatives which possess an aldehyde group are produced during incubation and highly unsaturated fatty acids may generate polar aldehydes after oxidative cleavage of the lipid chain.

Change in the Composition of Liposomal Phospholipids The contents of PC after storage were determined by the enzymatic method (Table 6). The value of PC including both peroxidized PC and normal PC did not change significantly, indicating that the carbonyl compounds produced during storage have PC structures. These PCs may contain acyl residues possessing an aldehyde functional group in the ω-position.

Discussion

Long-term stability at a wide range of temperatures is necessary for the practical application of liposomes. However, little has been reported about the decomposition of liposomes during long-term storage. In this work, we have investigated in detail the pH change in liposomal dispersions during storage as this is one of the most important factors for long-term stability. We have shown that peroxidation of unsaturated fatty acids is involved in the process of pH lowering in liposomal dispersions during storage. Hydrogenated, or partly hydrogenated, PC has been commonly used as a material to prepare liposomes but, because these PCs generally contain unsaturated fatty acyl chains as contaminants, the long-term stability of these liposomes is questionable. When phospholipids

containing unsaturated fatty acyl chains as contaminants are used for liposomes, peroxidation of unsaturated fatty acids in the process of pH lowering in liposomal dispersions will be observed irrespective of the preparation method, particle size and lamellarity of the liposomes. The incorporation of α-tocopherol effectively prevented the pH lowering, but egg PC liposomes with α-tocopherol still turned yellowish during storage even if bubbled with nitrogen.

These results indicate that hydrogenated PC with a low iodine value and a low POV, or synthetic PC such as DPPC, is suitable for medical applications which may involve prolonged storage.

In the present study, we have shown that peroxidation of phospholipids and the subsequent production of carbonyl compounds occur when liposomes composed of egg PC are incubated for a long period, leading to a lowering of the pH of the liposomal dispersion. The amount of carbonyl compounds correlates well with the pH of the egg PC liposomal dispersion after storage, whereas the amount of TBA-RS does not. About 0.8 μmol of carbonyl compounds per μmol of PC is formed after 12 days' storage and this greatly exceeds the amount of TBA-RS (about 2.0 nmol/μmol PC, Fig. 2c). When the formation of carbonyl compounds is suppressed by bubbling nitrogen or by the addition of antioxidants, pH lowering is no longer observed.

It is well known that many oxidative products are produced during lipid peroxidation. The monohydroperoxides, the first products of peroxidation, are rather unstable and easily decomposed into other products. The monohydroperoxides are known to be converted into various products containing polar and nonpolar aldehydes (carbonyl compounds) by successive scission, rearrangement and oxidation reactions.[9] The production of these carbonyl compounds may account for the lowering of the pH of liposomal dispersions.

From the results on the distribution of carbonyl compounds and lipid analysis after storage, the carbonyl compounds formed appear to be derived mainly from acyl residues of PC, and small amounts of polar aldehydes (peroxidation products of unsaturated acyl chains of PC) are released from membranes. We also detected non-polar or polar fatty acids which may be further oxidized products of carbonyl compounds in the liposomal dispersions. Small amounts of polar fatty acids (C4, C6, C8), non-polar fatty acids (C16, C18, *etc.*) and structurally unknown non-polar fatty acids, extracted from the liposomal dispersions with chloroform–methanol, were detected. Further studies are needed to determine precisely which non-polar or polar carbonyl compounds affect the pH of the liposomal dispersions during storage and these are in now progress.

References

1) Papahadjopoulos D. (ed.), "Liposomes and Their Uses in Biology and Medicine," Vol. 308, New York Acad. Sci., New York, 1978; Yagi K. (ed.), "Medical Application of Liposomes," Japan Scientific Soc. Press, Tokyo, 1986.
2) Gregoriadis G. (ed.), "Liposome Technology," Vol. 1, 2 and 3, CRC Press, Inc., Boca Raton, Florida, 1984; Gregoriadis G. (ed.), "Drug Carriers in Biology and Medicine," Academic Press, Inc., London, 1979.
3) Machy P., Leserman L. (eds.), "Liposomes in Cell Biology and Pharmacology," John Libbey, Inc., London/Paris, 1987.
4) Mezei M., Gulasekharam V., *Life Sciences*, **26**, 1473 (1980); Mezei M., "Topics in Pharmaceutical Sciences," ed. by Breimer D. D., Speiser P., Elsevier, Amsterdam, 1985, pp. 345—358.
5) Kikuchi H., Carlsson A., Yachi K., Hirota S., *Chem. Pharm. Bull.*, **39**, 1018 (1991).
6) Kikuchi H., Yamauchi H., U.S. Patent 4687661 (1987) [*Chem. Abstr.*, **102**, 137821 (1983)].
7) Buege J. A., Aust S. D., *Methods Enzymol.*, **52**, 302 (1978).
8) Lappin G. R., Clark L. C., *Anal. Chem.*, **23**, 541 (1951).
9) Esterbauer H., Cheeseman K. H., Dianzani M. U., Poli G., Slater T. F., *Biochem. J.*, **208**, 129 (1982).