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Hydrolysis of Partially Saturated Egg Phosphatidylcholine in Aqueous Liposome Dispersions and the Effect of Cholesterol Incorporation on Hydrolysis Kinetics

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Abstract—Hydrolysis kinetics of partially hydrogenated egg phosphatidylcholine (PHEPC) were studied as a function of pH, temperature, buffer concentration, ionic strength, and the effect of cholesterol incorporation. Results showed that PHEPC has a maximum stability at around pH 6·5. General acid base catalysis was observed for acetate, HEPES and Tris buffers. Increasing the ionic strength of the buffer solutions did not influence the hydrolysis kinetics. The relationship between the observed hydrolysis rate constants and the temperature could adequately be described by the Arrhenius equation. Incorporation of cholesterol did not affect the hydrolysis kinetics. This result indicates that the hydrolysis kinetics of PHEPC do not depend on the changes in bilayer rigidity induced by cholesterol incorporation. Cholesterol is stable under the experimental conditions used in this study; no changes were observed in cholesterol concentration over the experimental time interval.

Liposomes, phospholipid vesicles which form spontaneously in an aqueous environment, have been extensively studied as drug carrier systems. The physical and chemical instability of certain aqueous liposome dispersions on storage causes difficulties in their formulation as pharmaceutically acceptable products. Therefore, as a part of pharmaceutical formulation studies, assessment and manipulation of the chemical stability of liposomes is an important issue.

In an aqueous liposome dispersion, both saturated and unsaturated phospholipids undergo hydrolytic degradation to form lysophospholipids and fatty acids. These lysophospholipids are also subject to further hydrolysis to glycerophospho compounds and fatty acids (Kensil & Dennis 1981; Frøkjaer et al 1984; Kemps & Crommelin 1988; Grit et al 1989, 1993). The hydrolytic degradation products may change the properties of liposomal bilayers, such as rigidity. Furthermore, the physical stability of lipsome-based formulations measured as changes in the particle size and its distribution and retention of the encapsulated drug might also be affected by hydrolytic degradation products (Inoue & Kitagawa 1974). Up until now, the means to minimize hydrolysis reactions in aqueous dispersions have been limited and concentrate around selecting the optimum pH, buffer and temperature or, alternatively, freeze-drying of liposomes has been proposed as a rather drastic solution of the problem of hydrolytic reactions (Özer et al 1988).

In addition to hydrolysis, phospholipids with unsaturated acyl chains, are also subject to oxidation which may also affect the permeability of the bilayers and the in-vivo performance of the liposomes (Smolen & Shohet 1974; Konings 1984). Oxidation of phospholipids in an aqueous liposome dispersion can be minimized by addition of antioxidants such as tocopherols and butylhydroxytoluol, and proper preparation conditions (e.g. exclusion of oxygen and protection against light). Recently, a protective effect of buffers such as HEPES and Tris against lipid peroxidation has been reported by Fiorentini et al (1989).

Although cholesterol is not essential for the formation of liposomes, it is often used in pharmaceutical liposome formulations. It tends to increase retention of water-soluble drugs, it counteracts lipid phase transition and increases resistance to in-vivo liposome degradation (Papahadjopoulos et al 1973; Kirby et al 1980; Senior & Gregoriadis 1982). Different mechanisms of interaction between bilayer-forming phospholipids and cholesterol have been proposed; one of them is the formation of hydrogen bonds between the 3-hydroxyl group of cholesterol and the fatty acyl esters of phospholipids at both sn-1 and sn-2 positions (Brockerhoff 1974; Huang 1977). Moreover, it is well known that incorporation of cholesterol increases the rigidity of 'fluid state' liposomal bilayers (Fiorini et al 1989). If any physicochemical interaction exists between two bilayer components, an effect on the hydrolysis kinetics of phospholipids may also be expected. A direct interaction between the cholesterol 3-OH group and the acyl ester groups of phospholipids, being the most liable to hydrolysis, is likely to reduce hydrolysis rate constants as water molecules are hindered in approaching the ester bonds.

Egg yolk phosphatidylcholine (EPC) is an attractive raw material for the development of liposome-based pharmaceuticals because of its availability and low cost. However, EPC is a mixture of molecular species of PC differing in fatty acyl chains which includes a considerable amount of polyunsaturated fatty acids, such as arachidonic (C20:4) and decasohexanoic (C22:6) acids. These acids are more sensitive to oxidative degradation than less unsaturated species. Partially hydrogenated EPC (PHEPC, with an iodine value of 40) is prepared by catalytic hydrogenation of natural EPC. Its decreased sensitivity to oxidation was demonstrated with an accelerated oxidation test by Lang et al (1990). Further-

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more, PHEPC does not show any gel-to-liquid phase transition above 0°C. Therefore, changes in the physicochemical properties of liposomes due to a phase transition will not occur (Chapman 1975; Lang et al 1990).

In this study, hydrolysis kinetics of PHEPC were investigated as a function of pH, temperature, buffer concentration and ionic strength. The data were analysed to assess the catalytic effects of different buffer species used and compared with those obtained for natural (Grit et al 1989) and saturated soybean PC (Grit et al 1993). Furthermore, the effect of cholesterol addition on hydrolysis kinetics of PHEPC was determined in PHEPC-cholesterol containing bilayers.

Materials and Methods

Materials

PHEPC (iodine value = 40) originated from Asahi Chemical Industry Co. Ltd (Ibarakiken, Japan) and cholesterol was obtained from Croda (Edison, NJ, USA) and used as received. PHEPC consisted of 98·2% PC, 1·0% sphingomyelin, 0·7% lysophosphatidylcholine (LPC) and 0·4% water. The fatty acid composition of PHEPC as determined by Lang et al (1990) was as follows: (%) myristic acid 0·8, palmitic acid 31·9, palmitoleic acid 1·1, stearic acid 13·6, oleic acid 40·6, linoleic acid 4·2, C20:0 0·3, C20:1 3·2, C22:0 0·4, and C22:1 3·8. All solutions were prepared with double-distilled water. Other reagents used were of analytical grade.

Buffer solutions

The following aqueous buffer solutions were used for the kinetic studies: pH 4·0–5·0 acetate buffer, pH 5·6–6·5 citrate buffer, pH 6·8–7·5 HEPES buffer and pH 8·0–9·0 Tris buffer. The buffer concentration was in the range from 0·05 to 0·15 M. The pH of the liposome dispersions was measured with a glass electrode and a pH meter (Type CG 817 T, Schott Geräte, Germany) at the temperature of the experiment. The ionic strength of the buffer solutions was not adjusted in those experiments where the effect of pH, buffer concentration or temperature was investigated. The effect of ionic strength (adjusted by addition of NaCl) on the hydrolysis kinetics was investigated at pH 4·5, 6·5 and 8·0 in the range from 0·02 to 0·50.

Preparation of liposome dispersions

Liposome dispersions were prepared by the film method of Szoka & Papahadjopoulos (1980). After formation of a lipid film from a solution of PHEPC and cholesterol, if present, in chloroform in a round-bottom flask using a rotary evaporator at 50°C, the film was left overnight under reduced pressure. The initial PHEPC concentration was 20 μmol mL^{-1} and the cholesterol concentration varied from 0 to 40% (on a molar basis) of PHEPC. The film was hydrated at 50°C with the appropriate buffer solution and the pH of the dispersion was measured and adjusted, if necessary. The liposomes used were unextruded. The dispersions were stored overnight in a refrigerator, and subsequently, the pH values of the dispersions were measured at the temperature of the experiment and adjusted, if necessary. For all samples the pH was monitored over the experimental time interval; no significant changes were observed (< 0·1 pH units).

Kinetic measurements

The prepared liposome dispersions were filled into 1 mL ampoules under a nitrogen atmosphere and sealed. Ampoules were stored either in a constant temperature water bath (40 and 50°C) or in a constant temperature cabinet (60 and 70°C) which was equilibrated to the required temperature before use. Samples were taken after appropriate time intervals and analysed by HPLC. Samples were stored in a freezer (−20°C) until analysis. All kinetic measurements were performed in duplicate.

HPLC analysis

The HPLC system consisted of a solvent delivery system (Type 4000, Kratos, Ramsey, NJ, USA), a WISP 710 B automatic sampler, a model 410 differential refractometer (RI detector, both from Waters Associates, Milford, MA, USA) and a UV–VIS variable wavelength detector (Spectroflow 773, Kratos, Ramsey, NJ, USA). Quantitative analysis was based on peak areas while peak area measurement was carried out by an integrator (Type 3390 A, Hewlett Packard, Avondale, PA, USA).

PC and LPC were analysed as described earlier (Grit et al 1991). Samples for the HPLC analysis were prepared by dilution of the liposome dispersions in methanol; 20 μL of the diluted liposome sample was directly injected into the system. The analysis was performed in duplicate.

Cholesterol was determined using the procedure described by Lang (1990) with some slight modifications. Separation of cholesterol from its oxidative degradation products, PC and hydrolysis products of PC was achieved on a non-endcapped Spherisorb S-5 ODS-1 column (25 × 0·46 cm i.d.) with a mobile phase of 100% HPLC grade methanol at a flow rate of 1·5 mL min^{-1}. The column was operated at ambient temperature. Detection was carried out at 207 nm. Sample preparation was as described above.

Analysis of glycerophosphocholine (GPC) and its phosphate containing hydrolysis products

Since liposomes in partially hydrolysed samples were not successfully precipitated by centrifugation (30 min, 2700 *g*), judged on the basis of the turbidity of the obtained supernatant, phospholipids were extracted (Bligh & Dyer 1959). Afterwards, phosphorus was determined in the methanol–water phase as described by Fiske & Subbarow (1925). PC and LPC contents of the methanol-water phase were below the detection limit of the HPLC method (Grit et al 1991) and were neglected in the calculations.

Fluorescence polarization measurements

Membrane fluidity of liposome preparations was determined by measuring steady-state fluorescence polarization in a luminescence spectrophotometer (LS 50, Perkin-Elmer Ltd, Buckinghamshire, UK) as described by van Blitterswijk et al (1981). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as a fluorescent probe. In this method, the liposome dispersion was diluted to a phospholipid concentration of ~1 mM with the appropriate buffer solution and 5 μL DPH solution (2×10^{-4} M) in tetrahydrofuran was added to 3 mL of the diluted dispersion. After 1 h incubation at ambient temperature and in the dark, fluorescence polarization was measured at an excitation wavelength of 365 nm and an emission

wavelength of 430 nm. Light scattering was corrected with an appropriate non-DPH-containing liposome dispersion. All measurements were carried out at 60°C. Fluorescence polarization (P) was calculated as:

$$P=(I-I_{\perp})/(I+I_{\perp}) \quad (1)$$

where I is the intensity of the emitted light parallel to the direction of the excitation light and $I_{\perp}$ is the intensity of the part of the emitted light perpendicular to the excitation light.

Results

Order of the reaction

The disappearance of PHEPC in buffered dispersions followed pseudo first-order reaction kinetics within the experimental time frame at all pH and temperature values studied. This is indicated by the linearity ($r \geqslant 0{\cdot}99$) of the semilogarithmic plots of remaining PHEPC vs time (Fig. 1). These are typical plots obtained under widely differing experimental conditions. From the slopes of these straight lines, observed pseudo first-order hydrolysis rate constants (k_{obs}) were obtained. Reaction kinetics were not affected by incorporation of cholesterol into PHEPC bilayers.

In Fig. 2 decrease in the concentration of PHEPC and the formation of hydrolysis products are presented in a pH 4·0 buffer (C = 0·05 M) at 70°C. The lysophosphatidylcholine (LPC) concentration is presented as the sum of the two isomers of LPC, namely sn-1 and sn-2 LPC. sn-1 LPC was shown to be the main LPC formed as a result of PHEPC hydrolysis; it is at the same time a degradation product of sn-2 LPC via acyl migration (Plückthun & Dennis 1982). At every point, the mass balance for the phosphate-containing compounds in the samples was checked. The total phosphorus recovery in these samples was 100 ± 5%. Results presented in Fig. 2 were fitted with a nonlinear regression program in order to calculate the overall hydrolysis rate constants from PC to LPC and LPC to GPC. Hydrolysis rate constants of $1{\cdot}5 \times 10^{-6}\ s^{-1}$ and $2{\cdot}1 \times 10^{-6}\ s^{-1}$ were calculated for PC to LPC and LPC to GPC, respectively.

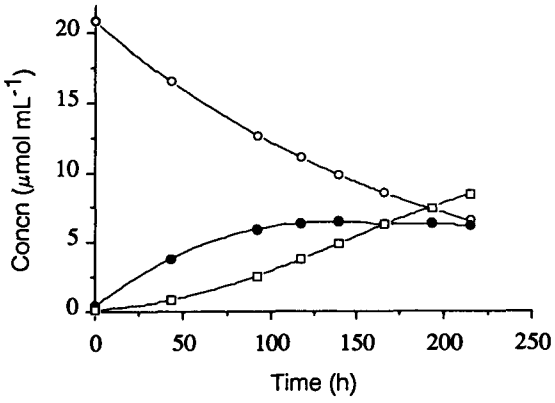

FIG. 2. Reaction time course of the hydrolysis of PHEPC in pH 4·0 acetate buffer at 70°C (C = 0·05 M). Each point represents the mean of two separate measurements. ○ PHEPC, ● LPC, □ GPC.

Influence of pH

The effect of pH on the hydrolysis of PC was investigated in the temperature range from 40 to 70°C. A possible pH change during the hydrolysis process due to the formation of the fatty acids may lead to results which are difficult to interpret. Therefore, it is necessary to keep the pH constant by using buffer solutions. Then, possible catalytic effects of the buffer components on the hydrolysis process have to be taken into account (Connors 1973). Under the conditions used in this study, the k_{obs} values obtained are the sum of the rate constants of the hydrolysis catalysed by water (k_0), proton (k_H), hydroxyl ions (k_{OH}) and the buffer species (k_{buffer}). The latter three are the second-order rate constants for the catalysed hydrolysis of PHEPC by the components of the buffer solutions. Thus, the k_{obs} value can be expressed as:

$$k_{obs}=k_0+k_H[H^+]+k_{OH}[OH^-]+k_{buffer}[buffer] \quad (2)$$

pH profiles (Fig. 3) were obtained at 0·05 M buffer concentration and in the temperature range from 40 to 70°C. In this figure the hydrolysis rate constants are not corrected for the buffer effects.

For each pH value, plots of k_{obs} against the buffer

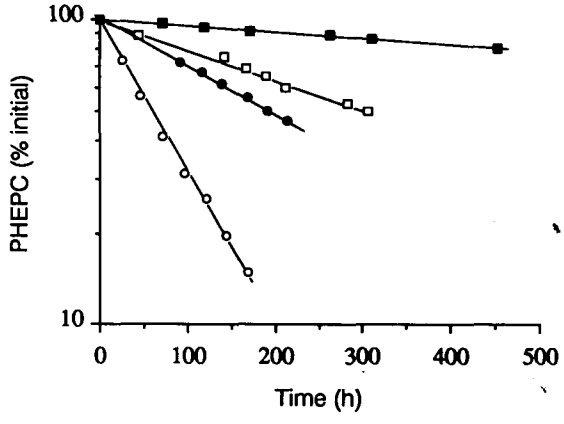

FIG. 1. Semilogarithmic apparent first-order plots for the hydrolysis of PHEPC. The lines were calculated by linear regression analysis. Each point represents the mean of two separate measurements. ○ pH 3·0 (+ 10% cholesterol, C = 0·05 M, 60°C), ● 4·5 (C = 0·05 M, 70°C), ■ 6·2 (C = 0·05 M, 70°C), □ 8·0 (μ = 0·30, 70°C).

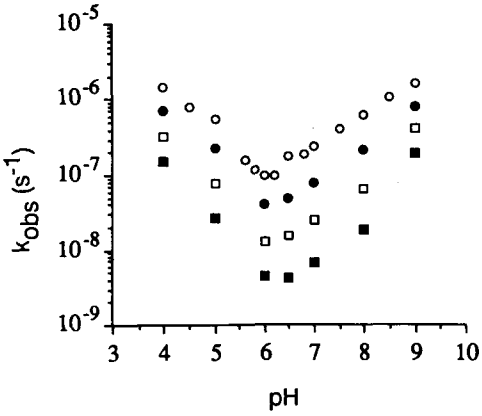

FIG. 3. The effect of pH on the hydrolysis of PHEPC (C = 0·05 M). Each point represents the mean of two separate measurements. ○ 70, ● 60, □ 50, ■ 40°C.

Table 1. Second-order rate constants ($M^{-1} s^{-1}$) for the catalysed hydrolysis of PHEPC at 70°C.

k_0[a]	$6{\cdot}2 \times 10^{-8}$
k_H	$1{\cdot}6 \times 10^{-2}$
k_{OH}	$2{\cdot}7 \times 10^{-1}$
k_{HAc}	$-1{\cdot}6 \times 10^{-6}$
k_{Ac}	$1{\cdot}1 \times 10^{-5}$
k_{Tris^+}	$7{\cdot}9 \times 10^{-6}$
k_{Tris}	$8{\cdot}7 \times 10^{-7}$
k_{HEPES^-}	$8{\cdot}2 \times 10^{-6}$
k_{HEPES}	$6{\cdot}1 \times 10^{-7}$

[a] in s^{-1}

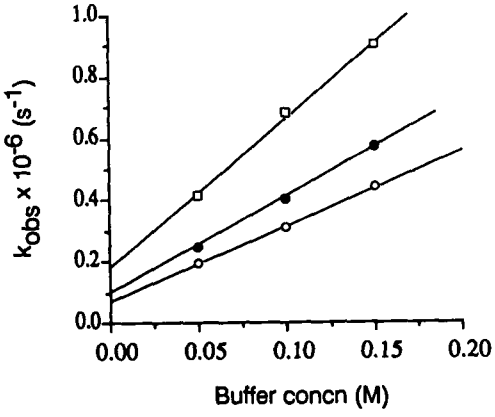

FIG. 4. The effect of buffer concentration on the hydrolysis of PHEPC in HEPES buffer at 70°C. Each point represents the mean of two separate measurements. The lines were calculated by linear regression analysis. ○ pH 6·8, ● 7·0, □ 7·5.

Table 2. The effect of ionic strength[a] on the hydrolysis of PHEPC at 70°C. Each number represents the mean of two separate measurements and the duplicate values were within 10% of each other.

	$k_{obs} \times 10^{-7}$ (s^{-1})		
Ionic strength	pH 4·5	pH 6·5	pH 8·0
0·02	8·3[b]	—	6·4[b]
0·10	8·3	—	6·1
0·20	8·1	—	6·4
0·29	—[c]	1·4[b]	—
0·30	8·2	—	5·3
0·33	—	2·2	—
0·40	—	1·8	—
0·50	—	1·9	—

[a] Ionic strength was adjusted by addition of NaCl. [b] Hydrolysis rate constants determined in 0·05 M buffer solution without NaCl. [c] Not determined.

concentration yielded a straight line with an intercept equal to the rate constant at zero buffer concentration (k'_{obs}). Plots of k'_{obs} against $[H^+]$ and $[OH^-]$ in the acidic and alkaline pH regions, respectively, yield straight lines with slopes equal to k_H and k_{OH}, respectively, and intercepts equal to k_0. This requires the assumption that at low pH where $k_{OH}[OH^-] \ll k_H[H^+]$ the catalytic effect of the OH^- ions can be neglected. Conversely, at high pH where $k_H[H^+] \ll k_{OH}[OH^-]$, the catalytic effect of the H^+ ions can be

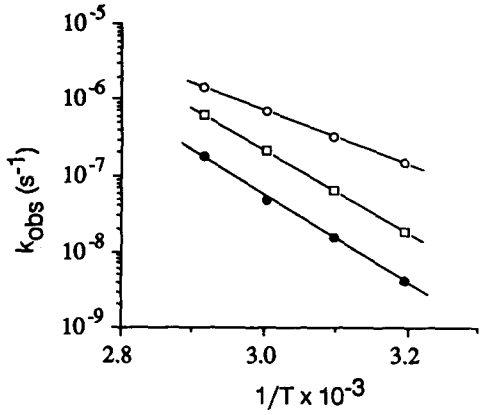

FIG. 5. The effect of temperature on the hydrolysis of PHEPC (C = 0·05 M). Each point represents the mean of two separate measurements. The lines were calculated by linear regression analysis. ○ pH 4·0, ● 6·5, □ 8·0.

neglected. The results obtained at 70°C are presented in Table 1.

Influence of buffer components

A linear relationship was found between the k_{obs} and the HEPES buffer concentration in the range 0·05–0·15 M for different pH values (Fig. 4). Similar plots were made for acetate and Tris buffers. The second-order rate constants for buffer catalysis were calculated from slopes of these plots as described earlier (Grit et al 1989), and are presented in Table 1.

Influence of ionic strength

The influence of ionic strength on the hydrolysis of PHEPC was studied at constant temperature, buffer concentration (C = 0·05 M) and at various ionic strengths (0·02–0·50) at pH 4·5 (acetate buffer), 6·5 (HEPES buffer) and 8·5 (Tris buffer). The ionic strength was adjusted by addition of NaCl. Results are presented in Table 2.

Influence of temperature

The effect of temperature on the hydrolysis of PHEPC was investigated in the temperature range of 40–70°C and in the pH range of 4·0–9·0 in buffer solutions at a buffer concentration of 0·05 M. The temperature dependency of the hydrolysis is described by the Arrhenius equation (Fig. 5). Since no phase transition from gel to liquid crystalline state takes place in the studied temperature range in PHEPC bilayers (Lang 1990), no discontinuity was expected in the Arrhenius curves (Grit et al 1993). Indeed straight lines were found with no discontinuity. Calculated Arrhenius parameters, such as activation energy and frequency factor, are presented in Table 3.

Influence of cholesterol incorporation and bilayer rigidity

The effect of cholesterol incorporation on the hydrolysis kinetics was investigated with 10–40% (on molar basis) cholesterol-containing liposomes at 60°C. The rigidity of PHEPC bilayers, measured by fluorescence polarization, increases with cholesterol incorporation. Fluorescence polarization (P) values at 60°C and at pH 8·0 are presented in

Table 3. Activation energies and frequency factors for the hydrolysis of PHEPC in aqueous liposome dispersions (C = 0·05 M).

pH	Activation energy (kJ mol^{-1})	Frequency factor (s^{-1})
4·0	68	$3·9 \times 10^4$
5·0	93	$8·6 \times 10^7$
6·0	94	$2·2 \times 10^7$
6·5	111	$1·5 \times 10^{10}$
7·0	105	$3·7 \times 10^9$
8·0	105	$8·6 \times 10^9$
9·0	64	$9·0 \times 10^3$

Table 4. Results of fluorescence polarization measurements on fresh and partially hydrolysed PHEPC-containing liposomes in pH 8·0 Tris buffer (C = 0·05 M) at 60°C.

Cholesterol content[a]	Fresh	Partially hydrolysed[b]
0	0·077	0·090
10	0·082	0·093
20	0·097	0·112
40	0·138	0·151

[a] Numbers of cholesterol molecules per 100 PHEPC molecules.
[b] In these samples around 40% of PHEPC was hydrolysed.

Table 5. The effect of cholesterol incorporation on the hydrolysis rate of PHEPC at 60°C (C = 0·05 M). Each number represents the mean of two separate measurements.

	$k_{obs} \times 10^{-7}$ (s^{-1}) Cholesterol content[a]			
pH	0	10	20	40
3·0	47	34	39	36
6·5	0·6	0·6	0·5	—[b]
7·0	1·0	0·8	0·9	0·9
8·0	3·3	3·3	3·7	3·3

[a] Number of cholesterol molecules per 100 PHEPC molecules.
[b] Not determined.

Table 4 for fresh and partially (40%) hydrolysed PHEPC samples. The P value, a measure for the bilayer rigidity, indeed increased with the cholesterol fraction. Slightly higher P values were found in partially hydrolysed samples (Table 4). Hydrolysis rates of PHEPC in cholesterol-containing liposomes are presented in Table 5 for different pH values. No significant change in the hydrolysis rate was observed.

Stability of cholesterol in PHEPC liposomes

The stability of cholesterol was assessed by the determination of the cholesterol concentration in fresh and in the final samples. No degradation products and negligible decrease in cholesterol concentration could be observed.

Discussion

The hydrolysis of PHEPC in aqueous liposome dispersions follows pseudo first-order reaction kinetics (Fig. 1). This is not affected by the formation of the hydrolysis products which results in degradation of the liposome structure at a later stage of hydrolysis as could be seen by the changes in turbidity and formation of oil-like drops (macroscopic observation).

Hydrolysis of PHEPC results in the formation of LPC in the first stage. The major LPC isomer formed was the sn-1 LPC isomer which was also formed via acyl migration from sn-2 LPC (Plückthun & Dennis 1982). LPC is further hydrolysed to GPC (Fig. 2). GPC is water-soluble and shows no amphiphilic properties, and, therefore, no interaction can be expected with liposomal bilayers. The fatty acids and LPC are the hydrolysis products which can interact with the bilayer because of their hydrophobic or amphiphilic properties. In many of the stability studies on liposomal formulations, the concentration of LPC is determined. On the basis of those results, the chemical stability of the formulation is judged (Hernández-Caselles et al 1990). The limited value of the conclusion drawn on the basis of such data is clearly shown in this study. The LPC concentration stabilized after a certain incubation period, due to its further hydrolysis. Similar results were reported for the alkaline (pH 12·7) hydrolysis of natural egg PC in mixed micelles and in liposomes (Kensil & Dennis 1981). Therefore, in order to determine the chemical stability of a liposome dispersion, analysis of all hydrolysis products as well as the initial product is recommended.

General acid-base catalysis was observed in the presence of buffer ions. There was a linear relationship between buffer concentration and hydrolysis rate in the range from 0·05 to 0·15 M. As could be seen in Table 1, the catalytic effects of the buffer ions are relatively small in comparison with the catalysis by proton and hydroxyl ions. Here again indications were found for a protective effect of acetic acid in good agreement with previously obtained results (Grit et al 1989, 1993).

No clear ionic strength dependency on the hydrolysis rate of PHEPC could be observed in buffer solutions at different ionic strengths, as would be predicted from the lack of net charge on PC.

Hydrolysis kinetics could adequately be described with the Arrhenius equation. No discontinuity in the Arrhenius curves was observed, indicating no transition takes place between the gel and liquid crystalline states. Since PHEPC contains a significant amount of unsaturated fatty acyl chains, it is, indeed, in the liquid crystalline state in the temperature range studied (Lang et al 1990).

The rigidity of the bilayer increased with its cholesterol content. However, the value of k_{obs} did not change. If a change occurs in the acceptability of the acyl ester bonds by water molecules in the presense of cholesterol, then this effect was not reflected in change kinetics (Zaslanky et al 1984).

On the basis of the results presented here, it can be concluded that hydrolytic degradation of PHEPC in liposomal bilayers is strongly pH-dependent; in formulating a liposome dispersion, the pH of the dispersion must be taken as a critical parameter to achieve a long shelf-life. The hydrolysis of phospholipids in aqueous liposome dispersions can not be monitored by measuring only the LPC content of the dispersions during storage, due to the further hydrolysis of LPC formed.

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References

Bligh, E. G., Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917

Brockerhoff, H. (1974) Model of interaction of polar lipids, cholesterol and proteins in biological membrane. Lipids 9: 645–650

Chapman, D. (1975) Phase transition and fluidity characteristics of lipids and cell membranes. Q. Rev. Biophys. 8: 185–235

Connors, K. A. (1973) Reaction Mechanisms in Organic Analytical Chemistry. Wiley, New York, pp 41–100

Fiorentini, D., Landi, L., Barzanti, V., Cabrini, L. (1989) Buffers can modulate the effect of sonication on egg lecithin liposomes. Free Rad. Res. Commun. 6: 243–250

Fiorini, R., Gratton, E., Curatola, G. (1989) Effect of cholesterol on membrane microheterogeneity: a study using 1,6-diphenyl-1,3,5-hexatriene fluorescence lifetime distributions. Biochim. Biophys. Acta 1996: 198–202

Fiske, C. H., Subbarow, Y. (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375–400

Frøkjaer, S., Hjorth, E. L., Wørts, O. (1984) Stability and storage of liposomes. In: Bundgaard, H., Bagger Hansen, A., Kofod, H. (eds) Optimization of Drug Delivery. Munksgaard, Copenhagen, pp 384–404

Grit, M., de Smidt, J. H., Struijke, A., Crommelin, D. J. A. (1989) Hydrolysis of phosphatidylcholine in aqueous liposome dispersions. Int. J. Pharm. 50: 1–6

Grit, M., Crommelin, D. J. A., Lang, J. K. (1991) Determination of phosphatidylcholine, phosphatidylglycerol and their lyso forms from liposome dispersions by high performance liquid chromatography (HPLC) using high sensitivity refractive index detection. J. Chromatogr. 585: 239–246

Grit, M., Underberg, W. J. M., Crommelin, D. J. A. (1993) Hydrolysis of saturated soybean phosphatidylcholine in aqueous liposome dispersions. J. Pharm. Sci. In press

Hernández-Caselles, T., Villaláin, J., Gómez-Fernández, J. C. (1990) Stability of liposomes in long term storage. J. Pharm. Pharmacol. 42: 397–400

Huang, C. H. (1977) A structural model for the cholesterol–phosphatidylcholine complexes in bilayer membranes. Lipids 12: 348–356

Inoue, K., Kitagawa, T. (1974) Effect of exogenous lysolecithin on liposomeal membranes: its relation to the membrane fluidity. Biochim. Biophys. Acta 363: 361–372

Kemps, J. M. A., Crommelin, D. J. A. (1988) Chemische stabiliteit van fosfolipiden in farmaceutische preparaten. I: Hydrolyse van fosfolipiden in waterig milieu. Pharm. Weekbl. 123: 355–363

Kensil, C. R., Dennis, E. A. (1981) Alkaline hydrolysis of phospholipids in model membranes and the dependence on their state of aggregation. Biochemistry 20: 6079

Kirby, C., Clarke, J., Gregoriadis, G. (1980) Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem. J. 186: 591–598

Konings, A. W. T. (1984) Lipid peroxidation in liposomes. In: Gregoriadis, G. (ed.) Liposome Technology. Vol. 1, Boca Raton, CRC Press Inc, pp 139–161

Lang, J., Vigo-Pelfrey, C., Martin, F. (1990) Liposomes composed of partially hydrogenated egg phosphatidylcholines: fatty acid composition, thermal phase behaviour and oxidative stability. Chem. Phys. Lipids 53: 91–101

Lang, J. K. (1990) Quantitative determination of cholesterol in liposome drug products and raw materials by high performance liquid chromatography. J. Chromatogr. 507: 157–163

Özer, Y., Talsma, H., Crommelin, D. J. A., Hincal, A. A. (1988) Influence of freezing and freeze drying on the stability of liposomes dispersed in aqueous media. Acta Pharm. Technol. 34: 129–139

Papahadjopoulos, D., Jacobson, K., Nir, S., Isac, T. (1973) Phase transition in phospholipid vesicles: fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim. Biophys. Acta. 311: 330–348

Plückthun, A., Dennis, E. A. (1982) Acyl and phosphoryl migration in lysophospholipids: importance in phospholipid synthesis and phospholipase specificity. Biochemistry 21: 1743–1750

Senior, J., Gregoriadis, G. (1982) Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of cholesterol and phospholipid components. Life Sci. 30: 2123–2136

Smolen, J. E., Shohet, S. B. J. (1974) Permeability changes induced by peroxidation in liposomes prepared from human erythrocyte lipids. J. Lipid Res. 15: 273–280

Szoka, F., Papahadjopoulos, D. (1980) Comparative properties and method of lipid vesicles (liposomes). Annu. Rev. Biophys. Bioeng. 9: 467–508

van Blitterswijk, W. J., Van Hoeven, R. P., Van Der Meer, B. W. (1981) Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. Biochim. Biophys. Acta 644: 323–332

Zaslauky, B. Y., Borouskaya, A. A., Rogozhin, S. U. (1984) Effect of lipid composition on hydrophobic properties of liposomes. Mol. Cell. Biochem. 60: 131–136