Research Papers

Hydrolysis of phosphatidylcholine in aqueous liposome dispersions

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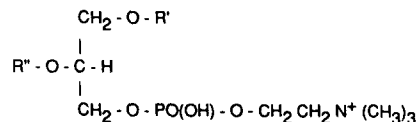
Summary

The hydrolysis of phosphatidylcholine was investigated as a function of pH, temperature, buffer concentration and buffer species. The hydrolysis rate of phosphatidylcholine increased with increasing concentration of the buffer species; there was a linear relationship between the buffer concentration and the observed rate constant. The pH profile at zero buffer concentration and at $72°C$ shows a minimum hydrolysis rate at about pH 6.5. The data were analysed to obtain detailed information on the effect of the buffer species used on the stability. The relationship between the observed rate constant and temperature could be described adequately by the Arrhenius equation.

Introduction

From a pharmaceutical point of view, it is important to demonstrate that drugs or dosage forms are sufficiently stable, so that they can be stored for a reasonable period of time without changing into an inactive or toxic form.

Liposomes are under investigation as drug carrier systems for their potential to improve the therapeutic index of drugs to be used e.g. in cancer chemotherapy or for the treatment of life-threatening parasitic, viral or microbial infections. Liposomes are vesicular structures build up of lipid bilayers. For therapeutic purposes usually phosphatidylcholine (PC) is used as the main (phospho)lipid component. Chemical decomposition of phospholipids (hydrolysis or oxidation) causes physical instability of the liposome dispersions and might therefore interfere with the introduction of liposomes in therapy (Inoue and Kitagawa, 1974, Smolen and Shohet, 1974 and Kibat and Stricker, 1985). Phospholipids can be hydrolysed to lyso-phospholipids; these lyso-phospholipids are also subject to further hydrolysis. 2-lysophospholipids are the main initial hydrolysis products in aqueous dispersions (Fig. 1) (Kemps and Crommelin, 1988).

$$CH_2 - O - R'$$
$$|$$
$$R'' - O - C - H$$
$$|$$
$$CH_2 - O - PO(OH) - O - CH_2 CH_2 N^+ (CH_3)_3$$

Fig. 1. Chemical structure of phosphatidylcholine (R' and R'' are the fatty acyl substituents) and 2-lyso-phosphatidylcholine (R' is the fatty acyl substituent and R'' is H).

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In this study, hydrolysis of PC as a function of pH, temperature and buffer concentration was investigated. The data were analysed to assess catalytic effects of the different buffer species used.

Materials and Methods

Materials

Soybean PC (Phospholipon 100) was obtained from Nattermann GmbH, Cologne, F.R.G. and used as received. PC consisted of 90% PC, 5.0% lyso-compounds, 5.0% free fatty acids and less than 0.1% water. The fatty acid composition of PC, determined by high performance liquid chromatography (HPLC), was as follows: 3.8% myristic acid, 18.2% palmitic acid, 4.2% stearic acid, 12.0% oleic acid, 61.8% linoleic acid. Other chemicals were of analytical grade. All solutions were prepared with double-distilled water.

Buffer solutions

The following aqueous buffer solutions were used for the kinetic studies: pH 4–5 acetate buffer, pH 5 to 6.5 citrate buffer and pH 7 to 9 Tris buffer. The pH was measured with a glass electrode and a pH meter (Type CG 817 T, Schott Geräte, F.R.G.). Ionic strengths of the buffer solutions were 0.068, 0.125, 0.200 and 0.300 and adjusted by manipulating the concentration of the buffer components.

Preparation of the liposome dispersions

PC liposome dispersions were prepared by the "film" method (Szoka and Papahadjopoulos, 1980). After formation of the phospholipid film in a round-bottom flask in a rotary evaporator at ~ 50°C, the film was left under reduced pressure overnight. It was hydrated at ~ 50°C with the appropriate buffer solution and the pH of the dispersion was measured and adjusted, if necessary. The initial PC concentration was 30 mM. Extrusion was carried out twice through a membrane filter with a pore size of 0.2 μm (Uni-pore, Bio-Rad, Richmond, CA, U.S.A.). The vesicle diameter, determined by dynamic light scattering using the Malvern PCS 2.4/2.3 software with a

Malvern 4600 apparatus (Malvern Ltd, Malvern, U.K.), equipped with a 25 mW helium/neon laser (NEC Corp., Tokyo, Japan), was around 0.19 μm. The dispersions were stored in the refrigerator overnight and extruded again the next day. The pH of the dispersions was also measured and adjusted between the extrusions; the pH was followed during the studies on degradation kinetics for all samples; no changes were observed.

Kinetic measurements

The prepared liposome dispersions were filled into 1 ml ampoules under nitrogen atmosphere in an LAF cabinet and sealed. Ampoules were stored either in a constant temperature water bath or a constant temperature cabinet, which were equilibrated to the required temperature prior to use. Samples were taken after appropriate time intervals and analysed by HPLC. Degradation kinetics was monitored at 40, 50, 60, 72 and 82°C.

HPLC analysis of PC

The HPLC analysis of PC was based on the method described by Nasner and Kraus (1981); it was slightly modified. The HPLC system consisted of a solvent delivery system type 6000A, a WISP 710 B automatic sampling unit (both from Waters Associates, Milford, MA, U.S.A.) and a variable wavelength detector (Model SF 773, Kratos, Ramsey, NJ, U.S.A.). The analytical (25 cm × 4.6 mm) column was filled with Lichrosorb SI 60 (10 μm particles) packing material (Merck, Darm-
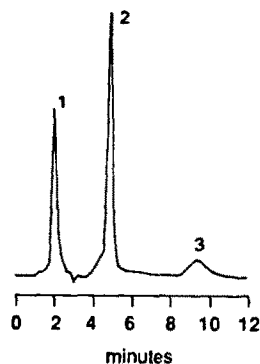

Fig. 2. HPLC chromatogram of phosphatidylcholine. For conditions see Materials and Methods. 1, solvent front + fatty acids; 2, phosphatidylcholine; 3, lyso-PC).

stadt, F.R.G.). PC separation was achieved with an eluent consisting of n-hexane–isopropanol–water (2:4:1, v/v) at a flow rate of 1 ml/min. Detection of PC was carried out at 206 nm. A typical example of a chromatogram showing a partly hydrolysed dispersion is presented in Fig. 2. Peak heights were used to quantify PC. Standard curves exhibited a linear response ($r > 0.999$) in the concentration range of 0.1–1 mM. The total phosphorus content of the ampoules was determined with the procedure of Fiske and Subbarow (1925).

Results

A typical example of the obtained data points during storage of PC liposome dispersions is presented in Fig. 3. The disappearance of PC in buffered solutions followed pseudo first-order kinetics. This is indicated by the linearity of the semilogarithmic plots of PC concentration vs time ($r \geq 0.99$). From the slopes of these straight lines the pseudo first-order rate constants (k_{obs}) were obtained.

The rate equation can be written as:

$$-\frac{d[PC]}{dt} = k_{obs}[PC] \qquad (1)$$

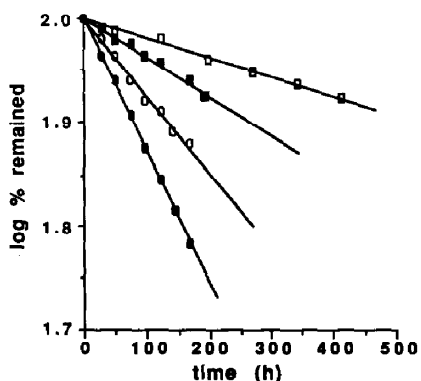The formation of fatty acids from the hydrolysis of PC tends to change the pH during storage.

Fig. 3. Semilogarithmic apparent first-order plots for the degradation of PC in pH 6.5 citrate buffer ($\mu = 0.068$). ($\bullet$, 82°C; $\circ$, 72°C; $\blacksquare$, 60°C; $\square$, 50°C). The lines were calculated by linear regression analysis.

Therefore, it is necessary to keep the pH constant by using buffer solutions. The possible catalytic effect of the buffer on the degradation process has to be taken into account (Connors, 1973). Substantial catalytic effects of the buffer components in the stability studies were reported by investigators for other substances (Beijnen, 1986; Carrey, 1987). To evaluate the contribution of the buffer species, the k_{obs} value can be expressed as:

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

where k_0 is the first-order rate constant for the degradation in water only, k_H and k_{OH} are the second-order rate constants for proton- and hydroxyl-catalysed degradation, respectively, and k_{buffer} is the sum of the second-order rate constants for the degradation catalysed by each of the buffer components.

The catalytic effects of the buffer components on the hydrolysis of PC were investigated at constant pH and temperature (72°C), but at different buffer concentrations. In this case, the term k_{buffer} [buffer] in Eqn. 2 is varied while the other terms are constant.

For each pH value, plots of k_{obs} against the buffer concentration yield a straight line with an intercept equal to the rate constant (k'_{obs}) at zero buffer concentration (Eqn. 3) and a slope equal to the second-order rate constant for catalysed degradation by the buffer components. These k'_{obs} values were used to obtain the pH profile of PC (Fig. 4).

$$k'_{obs} = k_0 + k_H[H^+] + k_{OH}[OH^-] \qquad (3)$$

For PC hydrolysis kinetics, plots of k'_{obs} against [H$^+$] and [OH$^-$] yield straight lines with the slopes equal to k_H and k_{OH}, respectively, and the intercepts equal to k_0. If k_{OH} [OH$^-$] $\ll k_H$ [H$^+$] then the catalytic effect of the OH$^-$ ions can be neglected. Conversely, if k_H [H$^+$] $\ll k_{OH}$ [OH$^-$] then the catalytic effect of the H$^+$ ions can be neglected.

In buffer solutions, the buffer concentration is the sum of the concentrations of the buffer species.
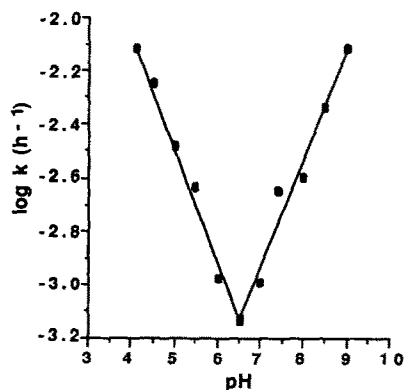
Fig. 4. Effect of pH on the degradation of PC at 72°C (buffer concentration = 0). The lines were calculated by linear regression analysis.
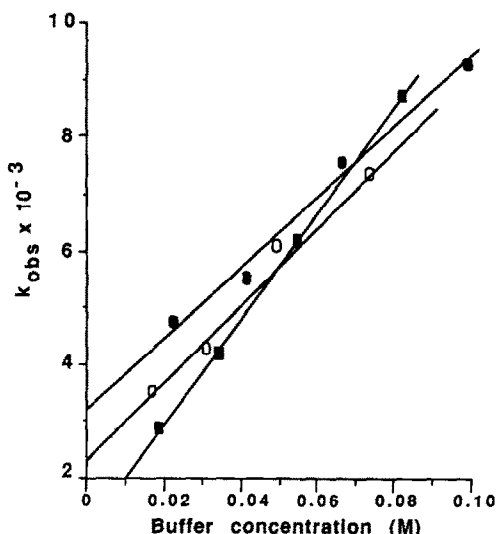

Fig. 5. Effect of the buffer concentration of the degradation of PC in citrate buffer at 72°C. (●, pH 5.0; ○, pH 5.5; □, pH 6.0). The lines were calculated by linear regression analysis. Each point represents the mean of at least two separate deter - minations.

The concentration of each buffer component is equal to the mole fraction (f) of the buffer component multiplied by the buffer concentration. Therefore, Eqn. 2 can be transformed into the following equations for acetate (HAc/Ac⁻, Eqn. 4), citrate (Ci^{3-}/Ci^{2-}, Eqn. 5) and Tris (Tris⁺/ Tris, Eqn. 6) buffer systems (Beijnen, 1986).

$$k_{obs} = k'_{obs} + \{(k_{Ac^-} - k_{HAc})f_{Ac^-} + k_{HAc}\}$$

$$[\text{buffer}] \tag{4}$$

$$k_{obs} = k'_{obs} + \{(k_{Ci^{3-}} - k_{Ci^{2-}})f_{Ci^{3-}} + k_{Ci^{2-}}\}$$

$$[\text{buffer}] \tag{5}$$

$$k_{obs} = k'_{obs} + \{(k_{Tris^+} - k_{Tris})f_{Tris^+} + k_{Tris}\}$$

$$[\text{buffer}] \tag{6}$$

Plots of k_{obs} against the buffer concentration yield straight lines with slopes equal to $(k_{Ac^-} - k_{HAc})f_{Ac^-} + k_{HAc}$, $(k_{Ci^{3-}} - k_{Ci^{2-}})f_{Ci^{3-}} + k_{Ci^{2-}}$ and $(k_{Tris^+} - k_{Tris})f_{Tris^+} + k_{Tris}$ in acetate, citrate and Tris buffers, respectively. Examples are shown in Fig. 5 for hydrolysis of PC in citrate buffer at 72°C. These plots were drawn for 3 pH values in acetate buffer, 4 pH values in citrate buffer and 5 pH values in Tris buffer. Plots of calculated slopes against the mole fraction of the Ac⁻, Ci³⁻ and Tris⁺ ions yield straight lines with intercepts equal to k_{HAc}, $k_{Ci^{2-}}$ and k_{Tris} and slopes equal to

$(k_{Ac^-} - k_{HAc})$, $(k_{Ci^{3-}} - k_{Ci^{2-}})$ and $(k_{Tris^+} - k_{Tris})$ in acetate, citrate and Tris buffers, respectively (Figure 6). The calculated rate constants for each buffer species are listed in Table 1.

The temperature dependence of the hydrolysis of PC was investigated in the pH range of 4–9 and temperature range of 40–82°C in the buffer solutions at the ionic strength of 0.068 (Fig. 7). The
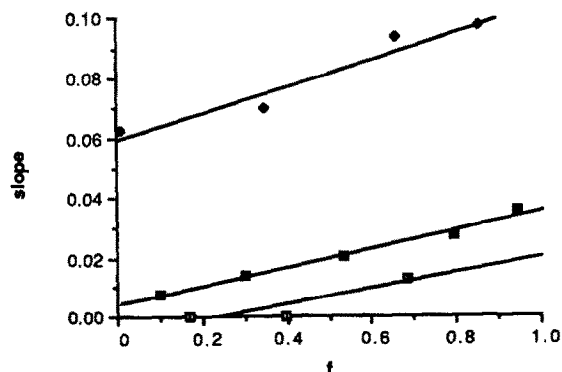

Fig. 6. The relationship between the mole fraction of the ions in the buffer solution and the slope of the buffer concentration-k_{obs} curves. (□, in acetate buffer; ◆, in citrate buffer; ■, in Tris buffer) The lines were calculated by linear regression analysis.

TABLE 1

Second-order rate constants for catalysed degradation of PC at 72°C

k_0	$8.5 \cdot 10^{-4} \pm 1.7 \cdot 10^{-4}$
k_H	$0.8 \cdot 10^{2} \pm 0.1 \cdot 10^{2}$
k_{OH}	$6.8 \cdot 10^{2} \pm 0.4 \cdot 10^{2}$
k_{Ac^-}	$2.0 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$
k_{HAc}	$-6.0 \cdot 10^{-3} \pm 4.4 \cdot 10^{-3}$
$k_{Ci^{-2}}$	$6.0 \cdot 10^{-2} \pm 4.3 \cdot 10^{-3}$
$k_{Ci^{-3}}$	$1.1 \cdot 10^{-1} \pm 1.2 \cdot 10^{-2}$
k_{Tris^+}	$3.6 \cdot 10^{-2} \pm 3.2 \cdot 10^{-3}$
k_{Tris}	$4.2 \cdot 10^{-3} \pm 1.3 \cdot 10^{-3}$

Constants expressed in $M^{-1} \cdot h^{-1}$, except for the first-order rate constant k_0 which is reported in h^{-1}.

effect of temperature on the decomposition is expressed by the Arrhenius equation (7):

$$\ln k_{obs} = \ln A - E_a / RT \qquad (7)$$

where A is the frequency factor, E_a is the activation energy, R is the gas constant and T is the absolute temperature.

Liposome dispersions are two-phase systems: a water phase and a bilayer phase can be discerned. The frequency factor for the liposome dispersions
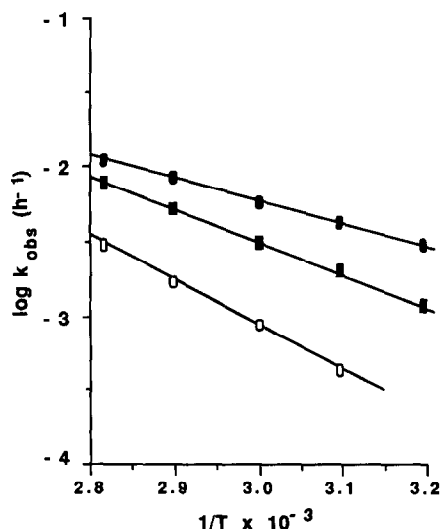

Fig. 7. Effect of the temperature on the hydrolysis of PC ($\mu = 0.068$). (●, pH 4.0; ○, pH 6.5; ■, pH 8.0). The lines were calculated by linear regression analysis. Each point represents the mean of at least two separate determinations.

TABLE 2

Activation energies (E_a), frequency factors (A), entropies of activation (ΔS^*) and probability factors (P) for degradation of PC as a function of pH ($\mu = 0.068$).

pH	E_a (kJ mol^{-1})	A (h^{-1})	ΔS^* (J K^{-1} mol^{-1})	P
4.0	29.7	$2.7 \cdot 10^2$	-130.4	$1.5 \cdot 10^{-7}$
5.0 *	48.5	$9.4 \cdot 10^4$	-81.5	$5.5 \cdot 10^{-5}$
5.0 **	42.1	$1.2 \cdot 10^4$	-99.0	$6.7 \cdot 10^{-6}$
6.0	43.3	$1.1 \cdot 10^4$	-99.5	$6.4 \cdot 10^{-6}$
6.5	57.2	$7.7 \cdot 10^5$	-64.1	$4.5 \cdot 10^{-4}$
7.4	42.3	$1.2 \cdot 10^4$	-98.8	$6.9 \cdot 10^{-6}$
8.0	41.9	$1.2 \cdot 10^4$	-99.0	$6.7 \cdot 10^{-6}$
9.0	39.0	$9.0 \cdot 10^3$	-101.1	$5.2 \cdot 10^{-6}$

Standard deviations were typically ca. 5%; * Acetate buffer. ** Citrate buffer.

was analysed in more detail to find out whether probability factor (P) differed significantly from hydrolysis kinetics in a homogeneous, one-phase system. The entropy of activation, ΔS^*, was calculated at 25°C from the frequency factor (A) obtained from Arrhenius equation by using Eqn. 8 and the probability factor was calculated from the ΔS^* values by using Eqn. 9 (Martin et al., 1983):

$$\Delta S^* = R(\ln A - \ln kT/h) \quad \text{and} \qquad (8)$$

$$P = e^{\Delta S^*/R} \qquad (9)$$

where k is the Boltzmann constant and h is Planck's constant. The obtained Arrhenius parameters are listed in Table 2.

Discussion

Hydrolysis of PC in liposome vesicles first results in lyso-PC and fatty acid formation. Further degradation to smaller fragments occurs in a later stage (Kemps and Crommelin, 1988). The initial degradation products are likely to interact with the bilayer. It was not investigated how much lyso-PC and fatty acids can be taken up by the liposomes before the vesicles disintegrate. Apparently, the degradation kinetics of PC were not affected by the presence of lyso-compounds or fatty acids as straight pseudo-first-order plots were

obtained. It should be mentioned that the pH tends to drop during storage unless proper buffers are used.

During storage of the dispersions under accelerated conditions a film was formed on the glass wall. This film could be redispersed by vortexing. The PC concentration, before and after vortexing, was similar. Therefore, the film was mainly composed of non-PC material. Degradation kinetics of PC were not affected by this third phase formation as no deviations from a straight line were observed.

General acid-base catalysis was observed by the effect of acetate, citrate and Tris ions. This is indicated by the slopes of the pH hydrolysis rate constant curves (slopes are equal to 0.4 for acid and base catalysis hydrolysis; Fig. 5) and the linear relationship between observed rate constant and buffer concentration. From Table 1 an increasing catalytic effect can be read with increasing anionic charge of the citrate ions. Negative catalysis (a protective effect) was observed for acetic acid as indicated by the minus sign in Table 1. Similar protective effects of the acetic acid on the stability of cyclosidomine have been described previously (Carrey, 1987). No attempt was made to disclose the mechanism of catalysis of protection.

The validity of Arrhenius law for the hydrolysis of PC was investigated in the temperature range between 40 and 82°C. Degradation kinetics could be adequately described by Eqn. 7. Frøkjaer et al. (1982) showed for distearoylphosphatidylcholine (DSPC) liposomes a break in the plot of ln k_{obs} vs $1/T$ around the transition temperature (T_c) of DSPC: 55°C (Frøkjaer et al., 1982). As mentioned in the experimental section, soybean PC is mainly composed of unsaturated fatty acids. The T_c of this type of phospholipids is about $-15°C$ (Szoka and Papahadjopoulos, 1980). This temperature range is above the transition temperature (T_c) of soybean phosphatidylcholine. Therefore, no break in the Arrhenius plot was observed (Fig. 7).

Further analysis of the frequency factor in the Arrhenius equation showed that probability factors of the hydrolysis kinetics of PC in liposome dispersions did not significantly differ from kinetics in homogeneous one-phase aqueous solutions (Frost and Pearson, 1961). Apparently the ester bonds (Fig. 1) are freely accessible for hydrolysis. From this study it can be decided that minimum degradation kinetics occur at pH 6.5 and that low buffer concentrations are favourable; $t_{90\%}$ values at pH 6.5 and at zero buffer concentration of 296 days at 6°C can be calculated. A lyso-PC content in PC vesicles of 10% (molar basis) increased the permeability dramatically (Inoue and Kitagawa, 1974; Kibat and Stricker, 1985). Work is in progress to investigate the effect of charge inducing agents and cholesterol on degradation kinetics and bilayer permeability.

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