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Acid-catalyzed plasmenylcholine hydrolysis and its effect on bilayer permeability: a quantitative study

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Abstract

This laboratory has previously shown (Anderson, V.C. and Thompson, D.H. (1992) Biochim. Biophys. Acta 1109, 33–42; Thompson, D.H., Gerasimov, O.V., Wheeler, J.J., Rui, Y. and Anderson, V.C. (1996) Biochim. Biophys. Acta 1279, 25–34), that plasmenylcholine (1-alk-1′-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PlsPamCho) liposomes release hydrophilic contents upon photooxidation or acid-catalyzed hydrolysis. We now report the kinetics and chemical mechanism of the acid-catalyzed reaction and its effect on calcein leakage rates. Hydrolysis of the plasmenylcholine vinyl ether linkage generates fatty aldehydes and 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine (lysolipid); HPLC and ^{1}H-NMR experiments establish that the former is readily air-oxidized to fatty acids, while the latter undergoes rapid acid-catalyzed rearrangement to 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine. Lysolipid formation obeys first order kinetics, yielding observed pseudo-first order rate constants that are pH-dependent. Bimolecular hydrolysis rate constants, k_{bi}, have also been determined. Calcein release rates from plasmenylcholine liposomes are strongly dependent on both the dihydrocholesterol (DHC) content and the extent of PlsPamCho hydrolysis within the bilayer. DHC-free plasmenylcholine liposomes (38°C, pH 2.5) require $< 5\%$ PlsPamCho hydrolysis to effect $> 50\%$ calcein release within 10 min. The presence of ≥ 25 mol% DHC, however, greatly reduces the observed calcein release rate; nearly 30% PlsPamCho hydrolysis is required to effect 50% calcein release over a 70-min period in 6:4 PlsPamCho/DHC liposomes. Bacteriochlorophyll *a*-sensitized photooxidation of plasmenylcholine liposomes also produces fatty aldehyde and another intermediate, tentatively described as 1-formyl-2-palmitoyl-*sn*-glycero-3-phosphocholine, that hydrolyzes to form the 1-hydroxy lysolipid. These results have important implications for the quantitative description of lysolipid effects on membrane permeability and on the design of triggerable liposomes for drug delivery.

Keywords: Controlled release; Lipid hydrolysis kinetics; Lipid oxidation; Lysophosphatidylcholine; Plasmalogen; Plasmenylcholine; Photooxidation; pH sensitive liposome; pH triggering; Phototriggering; Triggerable liposome; Vinyl ether hydrolysis

1. Introduction

The effect of lysolipids and fatty acids, formed by enzymatic cleavage or chemical hydrolysis, on phospholipid membrane properties has been extensively studied. The strong cytolytic and membrane-perturbing properties of lysophosphatidylcholines (1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholines or lysolipids),

Abbreviations: BChla, bacteriochlorophyll *a*; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ELSD, evaporative light-scattering detection; EPC, egg L-α-phosphatidylcholine; LUV, large unilamellar vesicles; lysolipid (lysoPC), 1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine; PlsPamCho, 1-alk-1′*Z*-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; RI, refractive index; ROS, reactive oxygen species; t_R, retention time

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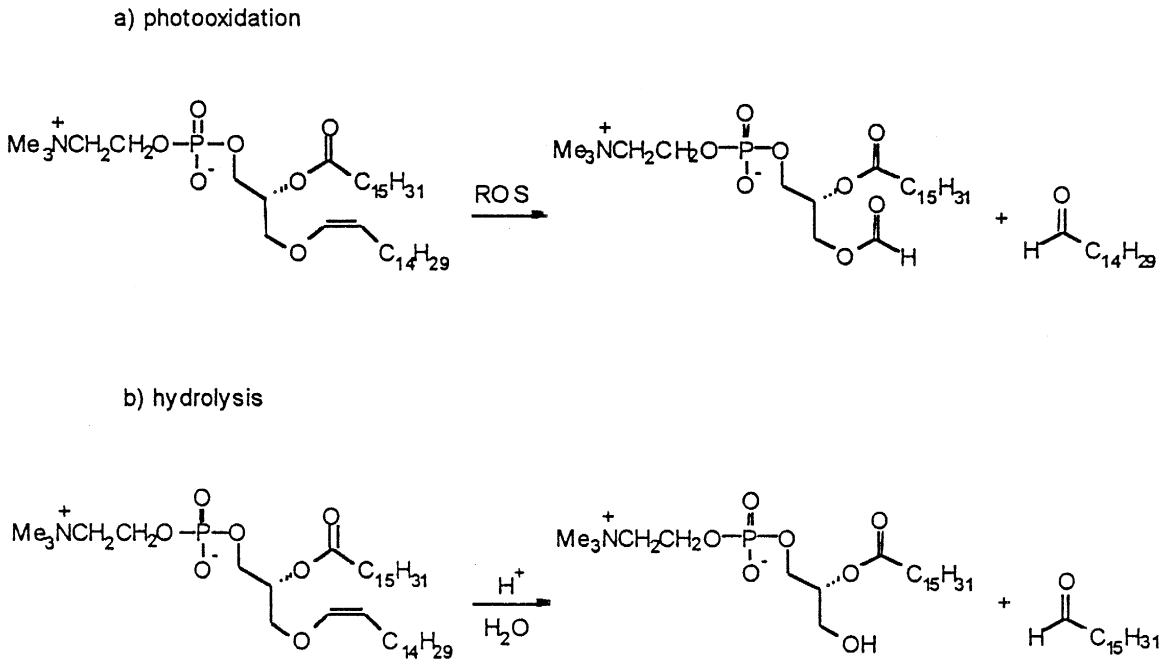
first described when lecithin suspensions and erythrocytes were treated with snake venom, are well known [1,2]. Interactions of lysophosphatidylcholine with model bilayer membranes, as well as their effect on the structure and properties of cellular membranes, have been of interest since Bangham [3] reported that the addition of lysolecithin to lecithin dispersions leads to a progressive disruption of lecithin 'spherulites' and to a 'beading' of lamellae. The permeability of lecithin liposomes also increases upon addition of exogenous lysolecithin [4,5]. Subsequent investigations established that (i) lysolecithin-induced leakage can be significantly suppressed by cholesterol incorporation within the membrane [4,6], (ii) liposomes become permeable at much lower lysophosphatidylcholine concentrations than that required for complete micellization of the bilayer [6], and (iii) phosphatidylcholine liposomes remain impermeable until critical concentrations of lysolipid and fatty acid are generated within the membrane as a result of either enzymatic [7] or chemical [8] hydrolysis. The latter findings are consistent with earlier observations that mixtures of lysolecithin and fatty acid can form stable bilayers [9].

Lysolipids, a ubiquitous component of biological membranes [10,11], are important intermediates in lipid metabolism and are known to be involved in the regulation of many membrane-bound enzymes [12,13]. Accumulated evidence also suggests that the surface activity of lysolipids may be more important than specific lysolipid–protein interactions, since their presence can exert profound effects on the structure, fluidity, permeability, and intrinsic curvature of membrane bilayers [14]. Lysolipid interaction with bilayers, relevant to the general problem of bilayer stability in the presence of surfactants, affects the pharmacological and toxicological properties of liposomes [15,16]. Poole and coworkers [17] have reported that lysolecithin added exogenously to a cell culture can facilitate cell fusion. Additional studies by Chernomordik and coworkers [18] have shown that membrane fusion between vesicles and bilayer lipid membranes can be either promoted or inhibited, depending upon the physical location of lysolipids present in the contacting membranes.

Our interest in the interactions of lysolipids with bilayer membranes was stimulated by our finding that contents release can be triggered by photooxidation or hydrolysis of plasmalogen liposomes [19–22]. Plasmalogens (formally, plasmenylcholines or 1-alk-



Scheme 1. Plasmalogen degradation pathways.

1′*Z*-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholines; PlsPamCho) are naturally occurring glycerophospholipids in which the *sn*-1-alkyl chain is connected via a *Z*-vinyl ether bond rather than an acyl linkage. The accumulation of lysolipid and other single-chain surfactants within the plasmenylcholine liposome membrane as a result of hydrolytic or photooxidative processes [23–25] was proposed as the key event responsible for liposome triggering and morphological transformations (Scheme 1) [20–22]. These studies, however, did not quantitatively correlate the degree of plasmenylcholine degradation required with the rates of liposomal contents leakage. We now report the kinetics of plasmenylcholine hydrolysis in liposome solution and its relationship to the kinetics of calcein leakage. Plasmenylcholine hydrolysis was chosen as a convenient experimental system, since the rates of plasmenylcholine decomposition and contents release can be readily varied by adjusting solution conditions such as pH and temperature. This confers a greater degree of kinetic control over the triggering chemistry since reactive oxygen species (ROS)-mediated decomposition of both plasmenylcholines and sensitizer are competing reactions in the photoinduced release process. The data presented below support the chemical mechanisms that were previously proposed and provide quantitative information about the interrelationship between hydrophilic contents release rates and the extent of plasmenylcholine decomposition at different pHs, temperatures, and dihydrocholesterol (DHC) content within the membrane. The relevance of this kinetic data to the photochemical liposomal triggering approach is also discussed.

2. Materials and methods

2.1. Materials

Egg L-α-phosphatidylcholine (EPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (both from Avanti Polar Lipids), bacteriochlorophyll *a* (BChla,

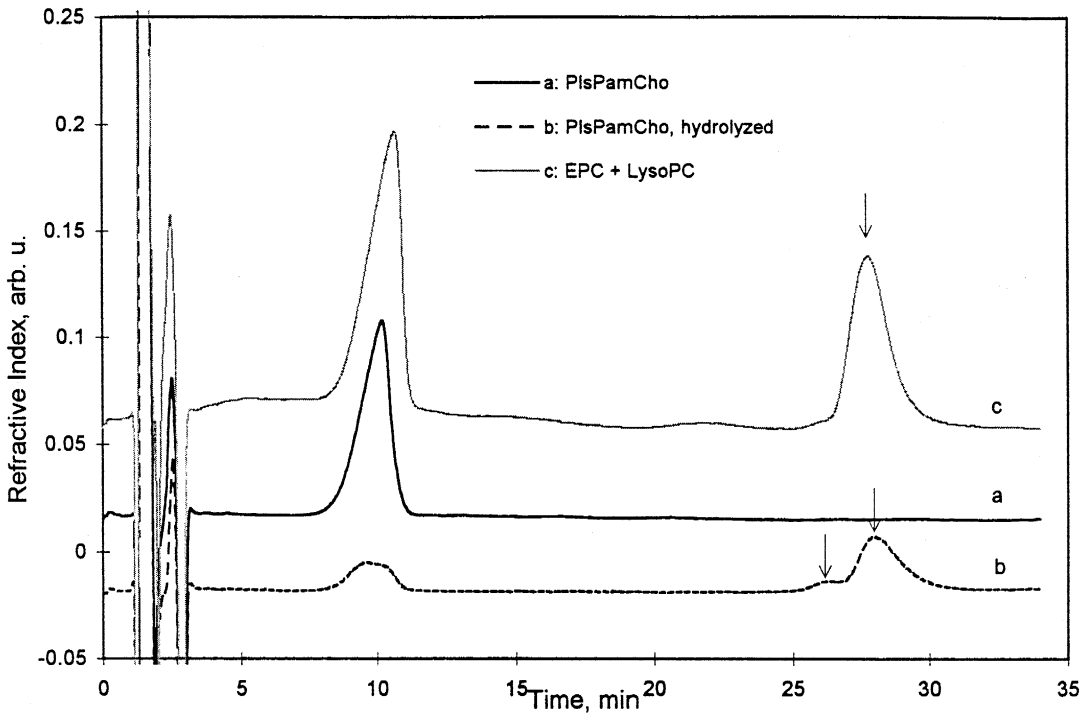


Fig. 1. HPLC chromatograms of plasmenylcholine hydrolysis products: (a) Bligh–Dyer extract of plasmenylcholine liposomes held at pH 7.4 for 48 h; (b) Bligh–Dyer extract of plasmenylcholine liposomes exposed to exhaustive hydrolysis at pH 2.5 for 3 h; (c) 1:1 mixture of EPC and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoPC) in chloroform solution. HPLC conditions: 3.9×300 mm μPorasil® column, 52:40:8 (v/v) hexanes/2-propanol/water eluent at 2.2 ml/min, RI detection.

Sigma), calcein (Sigma), (+)-dihydrocholesterol (DHC, Aldrich), tris-(hydroxymethyl)-aminomethane (Tris, Aldrich), and Triton X-100 (10% aqueous solution, Calbiochem) were used as received. Chloroform (spectrophotometric grade, Mallinckrodt) was filtered through anhydrous sodium carbonate to remove HCl, and stored over molecular sieves in the dark to minimize subsequent decomposition. All aqueous solutions were prepared using 18 MΩ-cm water from a Barnstead NANOpure system.

Semi-synthetic plasmenylcholine (PlsPamCho) was prepared from bovine heart lecithin (bovine heart L-α-phosphatidylcholine, Avanti Polar Lipids) as described elsewhere [19]. Normal phase HPLC (see Section 2.3 below) of the product did not reveal any compounds other than diradyl glycero-3-phosphocholines. Chromatographically-purified semisynthetic material had a retention time similar to EPC (Fig. 1a and b). Exhaustive hydrolysis of this material revealed that approximately 20% was non-hydrolyzable (Fig. 1c). This observation is in good agreement with the results of van den Bosch et al. [26] who reported a $\approx$ 66:6:33 ratio of diacyl:alkylacyl:alk-1-enylacyl-*sn*-glycerophosphocholines in bovine heart glycerophospholipid extract, corresponding to $\approx$ 18% non-hydrolyzable material. The major components ($\geq$ 80%) in the semisynthetic preparation, therefore, were 1-alk-1′*Z*-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholines, with the remainder being 1-alkyl-2-palmitoyl-*sn*-glycero-3-phosphocholines. All data were collected using a single batch of material.

2.2. Liposome preparation

Large unilamellar liposomes (LUV) were prepared by extrusion [27]. Pure plasmenylcholine liposomes were prepared from hydrated lipid powders; all other liposomes were prepared from lipid films evaporated from $CHCl_3$ as described previously [21]. Calcein (50 mM) was passively entrapped by hydrating the lipid in calcein solution (94 mg of calcein in 3 ml of 0.3 M NaOH, final solution pH = 12.5). After extrusion, the extraliposomal calcein was exchanged with 150 mM NaCl, using a 1 × 40-cm Sephadex G-50–150 column. HPLC analysis indicated that no appreciable PlsPamCho hydrolysis occurs during this LUV preparation protocol. Leakage of calcein from the liposomes prepared in this manner did not exceed 5% over 24 h when stored at temperatures from 4°C to 24°C. BChla-containing liposomes were prepared as described previously [21].

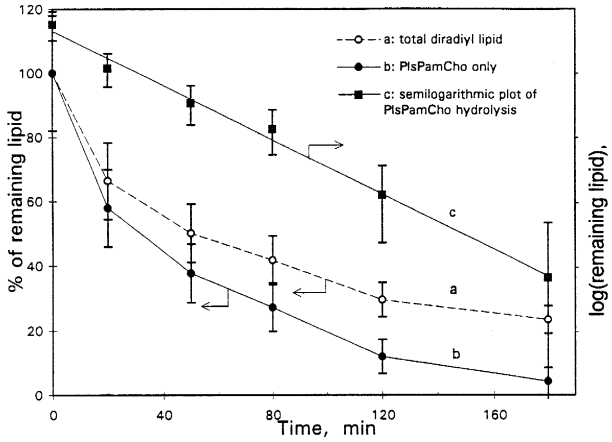


Fig. 2. Kinetics of plasmenylcholine liposome hydrolysis (pH 2.5, 38°C) measured as a: (a) decrease in the total diradylPC peak areas; (b) decrease in PlsPamCho content, assuming that the semi-synthetic material contains only 80% plasmenylcholine lipid, the balance (20%) being a mixture of non-hydrolyzable diradyl glycerophosphocholine lipids; (c) semilogarithmic plot of data shown in curve b.

2.3. HPLC analysis of plasmenylcholine degradation products

The HPLC procedure used was adapted from a method reported by Horrocks and coworkers [28]. HPLC analyses were performed using a μ-Porasil® 3.9 × 300 mm silica column, 52:40:8 (v/v) hexanes/2-propanol/water as eluent (2.2 ml/min), and differential refractometer (RI) or evaporative light scattering (ELSD, Sedex 55) detection. Lipids were extracted from the aqueous suspension using the Bligh–Dyer method [29] prior HPLC analysis. This method was reproducible for the extraction of diradyl lipids; however, it was considerably less efficient and reproducible for the extraction of lysolipids. The degree of hydrolysis as a function of time (Fig. 2a), therefore, was determined by using the areas of the diradylglycero-3-phosphocholine peaks as an internal standard (Fig. 1b, $t_R \cong 10$ min) in the extracts from samples withdrawn during the course of the reaction. Since the non-hydrolyzable fraction was approximately 20% of the total phospholipid content, the

following expression was used to calculate the degree of PlsPamCho decomposition:

$$x = [A(t) - A_0 \cdot 0.2]/A_0 \cdot 0.8$$

where $A(t)$ and A_0 are the areas of PlsPamCho HPLC peaks at time t, and $t = 0$, respectively, and where x is the mole fraction of PlsPamCho remaining. Plasmenylcholine hydrolysis kinetics calculated in this manner were exponential (Fig. 2b). Semilogarithmic plots ($\ln(x)$ vs. time; Fig. 2c) were used to determine the pseudo-first order hydrolysis rate constants; second order rate constants (k_{bi}) were calculated by dividing the observed pseudo first order rate constants by the proton activity of the bulk aqueous phase.

2.4. Liposome release assay

Aliquots of the liposome solution (50 μl) were quenched in 2 ml of 150 mM NaCl/20 mM Tris (pH 8.0) at various times during the course of the hydrolysis reaction and the calcein fluorescence intensity measured. The calcein release percentage was calculated as described previously [21].

2.5. Hydrolysis

Phosphate (pH 6.3 and 5.3), citrate (pH 4.3), and oxalate (pH 3.2) buffers (20 mM in 150 mM NaCl in all cases) were used for acid-catalyzed hydrolysis experiments above pH 3. Hydrochloric acid was used for reactions run at pH 2.3 and 1.5 [1]. Hydrolysis was initiated by mixing temperature-equilibrated liposomal solutions with isotonic acidic (1.5 ml) solutions and the reaction temperature maintained throughout the course of hydrolysis. Aliquots of these mixtures were withdrawn periodically for HPLC and calcein release analysis as described above.

2.6. Photolysis

Photolysis of BChla-containing liposomal solutions was performed using a SDL 820 diode laser (Spectra Diode Labs, San Jose, CA, $\lambda_{em} = 800$ nm) as described previously [21].

2.7. NMR measurements

Plasmenylcholine hydrolysis products were extracted from aqueous hydrolysis mixtures using the Bligh–Dyer method [29], evaporated, and dried under vacuum ($< 5 \cdot 10^{-2}$ mmHg) overnight. The extracts were then purified by HPLC and fractions corresponding to lysolipid and fatty aldehyde products were collected, evaporated, and dried under vacuum overnight. The dried, purified fractions were dissolved in $CDCl_3$ and the ^{1}H-NMR spectrum determined using a Varian Gemini-200 NMR spectrometer.

3. Results and discussion

3.1. Products of plasmenylcholine hydrolysis and photolysis

Acid-catalyzed hydrolysis of plasmenylcholine according to Scheme 1 results in the initial formation of 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine. The data shown in Fig. 1b, however, indicate that the main product of hydrolysis has a retention time identical to that of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Fig. 1c). This assignment was confirmed by ^{1}H-NMR experiments. An additional hydrolysis product with a slightly shorter retention time (Fig. 1c) and a peak area approximately 10% of the 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine peak is also observed. This component is presumably 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine, a lysolipid that is known to undergo facile acyl transfer under acidic conditions to form the more thermodynamically stable 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine [8,30]. These hydrolytic decomposition products are different from those initially expected from plasmenylcholine liposome photooxidation. The initial product of plasmenylcholine degradation by singlet oxygen should be 1-formyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (Scheme 1a), which may be further hydrolyzed to

[1] The pH increased by approx. 0.2 by the end of experiment in the case of the pH 2.5 experiments, due to the release of the alkaline calcein solution. The pH did not change appreciably during the pH 1.5 release experiments.

1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine and formic acid. The less polar species, 1-formyl-2-palmitoyl-*sn*-glycero-3-phosphocholine, would be expected to have a shorter retention time than the more polar 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine secondary hydrolysis product, but longer than the parent diradylphosphocholine. In fact, illumination of plasmenylcholine liposomes in the presence of the membrane-bound sensitizer bacteriochlorophyll *a* (BChla) results in the formation of two lipid degradation products, both having shorter retention times than 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Fig. 3). We believe these compounds are 1-formyl-2-palmitoyl-*sn*-glycero-3-phosphocholine ($t_R \cong 22$ min) and its hydrolysis product, 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine. Acyl migration (i.e. 1-hydroxy-lysolipid to 2-hydroxy-lysolipid isomerization) was presumably suppressed in this experiment by the neutral pH of the reaction medium.

The fatty aldehyde fragment from the plasmenylcholine hydrolysis coeluted with the solvent peak under our chromatographic conditions (Fig. 1b). This byproduct must be readily oxidized since the NMR spectrum of the compound isolated by HPLC did not contain an aldehyde resonance; signals at 2.35 ppm and 1.60 ppm, characteristic of methylene groups α- and β- to a carboxylic group, respectively, and the absence of an aldehyde formyl resonance strongly suggest that the intermediate aldehyde is air-oxidized to the corresponding carboxylic acid during HPLC isolation. This is further supported by the observation that the ^{1}H-NMR spectrum of the hydrolysis sample prior to HPLC isolation (i.e., immediately lyophilized after Bligh-Dyer extraction) did contain characteristic aldehyde proton resonances centered at 9.77 ppm.

3.2. Kinetics and mechanism of acid-catalyzed hydrolysis of PlsPamCho liposomes

3.2.1. pH and temperature dependence of PlsPamCho hydrolysis

Pseudo-first-order and apparent second-order rate constants for the hydrolysis of liposomal plasmenyl-

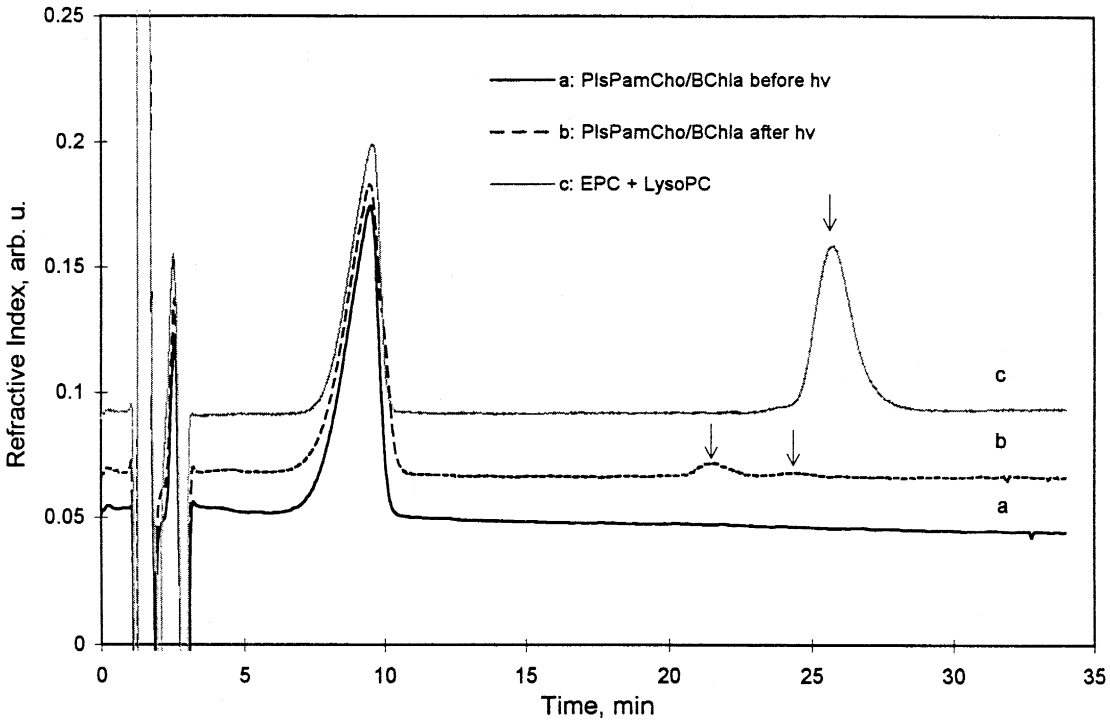


Fig. 3. HPLC chromatograms of plasmenylcholine photooxidation products: (a) Bligh–Dyer extract of PlsPamCho/BChla liposomes before photolysis; (b) Bligh–Dyer extract of the same liposomes after 30 min illumination with 800 nm light from an AlGaAs laser (incident light intensity = 500 mW, total dose = 900 J); (c) 1:1 mixture of EPC and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoPC) in chloroform solution.

Table 1
Pseudo-first-order (k_{mono}) and apparent second-order (k_{bi}) rate constants for plasmenylcholine hydrolysis in aqueous liposome solution at 38°C

pH	k_{mono}, s^{-1}	k_{bi}, $M^{-1} s^{-1}$ ($k_{bi} = k_{mono} / a_{H^+}$)
1.57	4.94 E-04	0.0183
2.53	1.65 E-04	0.0559
4.3	6.31 E-06	0.125
5.3	8.11 E-07	0.161

choline determined at different pHs appear in Table 1. Literature values for second order rate constants determined for the hydrolysis of some simple monosubstituted *Z*-vinyl ethers are shown in Table 2. These compounds can be viewed as model plasmenylcholines for the purposes of comparing their bimolecular hydrolytic rate constants with those determined for PlsPamCho hydrolysis. These second-order hydrolysis rate constants, k_{bi}, vary over a range of almost six orders of magnitude, from $7 \cdot 10^{-4}$ to $3 \cdot 10^{2}$ $M^{-1} s^{-1}$ in H_2O at 25°C, depending on the nature and number substituents [33]. Comparison of rate constants determined for simple vinyl ether model compounds that are close structural analogs of the plasmenylcholine vinyl ether linkage (observed k_{bi}s range from 0.25 to 1.2 $M^{-1} s^{-1}$, Table 2) with those measured for liposomal plasmenylcholines (k_{bi} = 0.018–0.16 $M^{-1} s^{-1}$, Table 1) suggests that the plasmenylcholine rates are slightly slower, but remain in reasonably good agreement with the model substrates.

The temperature dependence of plasmenylcholine hydrolysis (Fig. 4) indicated that the activation energy [2] for acid-catalyzed hydrolysis of PlsPamCho in 100 nm LUV was $E_{act} = 99 \pm 11$ kJ mol^{-1}. Although this activation barrier is noticeably higher than the values of E_{act} known for the hydrolysis of unsubstituted vinyl ethers (E_{act} = 57–69 kJ mol^{-1} [34,35]), this value is consistent with the observed slower rates of PlsPamCho hydrolysis compared with these same model compounds.

3.2.2. Mechanistic details of liposomal PlsPamCho hydrolysis

The rate-limiting step of vinyl ether hydrolysis typically involves protonation of the carbon β- to the vinyl ether oxygen [33,36]. Since in our case the vinyl ether moiety is located near the hydrophobic domain of the bilayer (i.e. $\approx$ 7–10 Å beneath the surface of the bilayer [37]), proton diffusion through the polar headgroup region must precede the rate-limiting protonation step. Diffusion, however, must not be the limiting step in our system since our observed activation energy is much higher than a typical activation energy for diffusion (between 13 and 20 kJ/mol in aqueous solutions [38]). The fact that the vinyl ether group is buried 7–10 Å below the bilayer surface may account for the smaller rate constants and higher activation energies for plasmenylcholine hydrolysis (Table 1) compared to those for other vinyl ethers (Table 2), since (i) the vinyl ether bond in liposomal plasmenylcholines is less available for protonation than the fully hydrated model compounds, and (ii) protonation of the β-carbon, which involves a sp^2 to sp^3 hybridization change, should correspond to a larger E_{act} since it requires reorientation of the hydrocarbon chain within the constraints of the bilayer matrix.

It is interesting to note that the measured values of the second order rate constants (k_{bi}) in Table 1 drop by an order of magnitude between pH 5.3 and 1.57, even though a constant k_{bi} would be expected for protonation as the rate-determining step. Since the β-carbon of the plasmenylcholine vinyl ether linkage is located close to the hydrocarbon core of the bi-

Table 2
Rate constants for the hydrolysis of some monosubstituted *Z*-vinyl ethers in aqueous solutions at 25°C

Vinyl ether	k_{bi}, $M^{-1} s^{-1}$
Methyl *Z*-propenyl ether	0.255 [a]
Ethyl *Z*-propenyl ether	0.48 [a]
Z-1-Propenyl methyl acetal	0.42 [b]
Isopropyl *Z*-propenyl ether	1.20 [a]

[a] Ref. [31]; [b] Ref. [32].

[2] The Arrhenius plot of the plasmenylcholine hydrolysis rate constant was linear both below and above the 35–43°C temperature region with a discontinuity in the vicinity of the bilayer melting point (37–38°C [19]). E_{act} was determined in the linear regions.

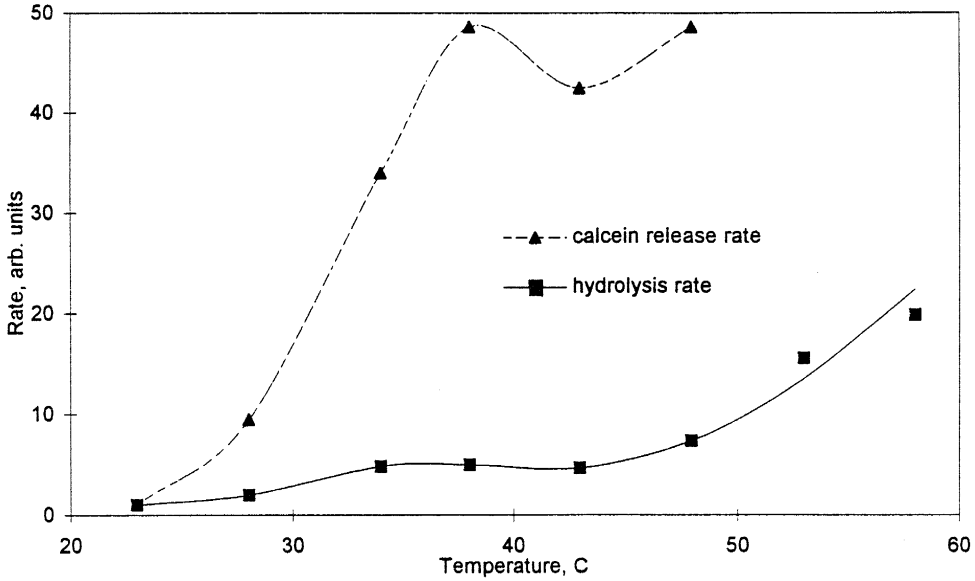


Fig. 4. Temperature dependence of plasmenylcholine hydrolysis rate constants (■) and calcein release rates (defined here as $1/t_{50\%\ release}$, ▲). Values of 1 were assigned to the rates at 23°C.

layer, the proton activity in this region must be different from that in bulk water. In fact, it must be non-linearly related to the a_{H^+} of bulk water, since the surface charge of the bilayer varies over this pH range due to protonation of the phosphate headgroup below pH 3 (originally reported for DPPC bilayers [39]). Headgroup protonation under our experimental conditions, therefore, may result in (i) a reduction in the bimolecular hydrolysis rate constant, k_{bi} (it should be noted that Kresge [31] has reported 100-fold reductions in bimolecular hydrolysis rate constants for several carboxy vinyl ether species due to protonation of neighboring carboxylate groups), and (ii) a decrease in the proton concentration in the bilayer environment due to an increase in the membrane surface potential that would electrostatically restrict further protonation (i.e. Gouy–Chapman–Stern effect) [40]. The decrease of k_{bi} at low pH (Table 1), therefore, can be explained by the involvement of one (or both) of these factors.

3.2.3. Influence of DHC on the rate of PlsPamCho hydrolysis

No significant differences were observed in the lipid hydrolysis rates for pure PlsPamCho liposomes and those containing up to 40 mol% DHC. These data suggest that the presence of DHC does not affect either the proton activity in the bilayer or the rate determining vinyl ether protonation step. Since DHC does not influence the rate of plasmenylcholine hydrolysis, it must play a different role in determining the permeability of PlsPamCho liposomes (see below).

3.3. Calcein release from PlsPamCho liposomes

3.3.1. Effect of plasmenylcholine hydrolysis on calcein release

Fig. 5a shows that the extent of calcein release strongly depends on the degree of plasmenylcholine hydrolysis. Calcein release occurs rapidly after the onset of plasmenylcholine hydrolysis in pure PlsPamCho liposomes ($t_{50\%\ release}$ occurs at approximately 3 min and $< 5\%$ PlsPamCho hydrolysis), whereas a much slower rate of release is observed in the case of 6:4 PlsPamCho/DHC liposomes ($t_{50\%\ release} = 63$ min, corresponding to $\approx 30\%$ PlsPamCho hydrolysis). The absence of a lag period in PlsPamCho liposome hydrolysis is in distinct contrast to the observed rates of contents permeation initiated by diacylphosphatidylcholine hydrolysis [7,8,41]. The basis for these differences may be attributed to the degree of hydrogen bonding that can occur between the hydrol-

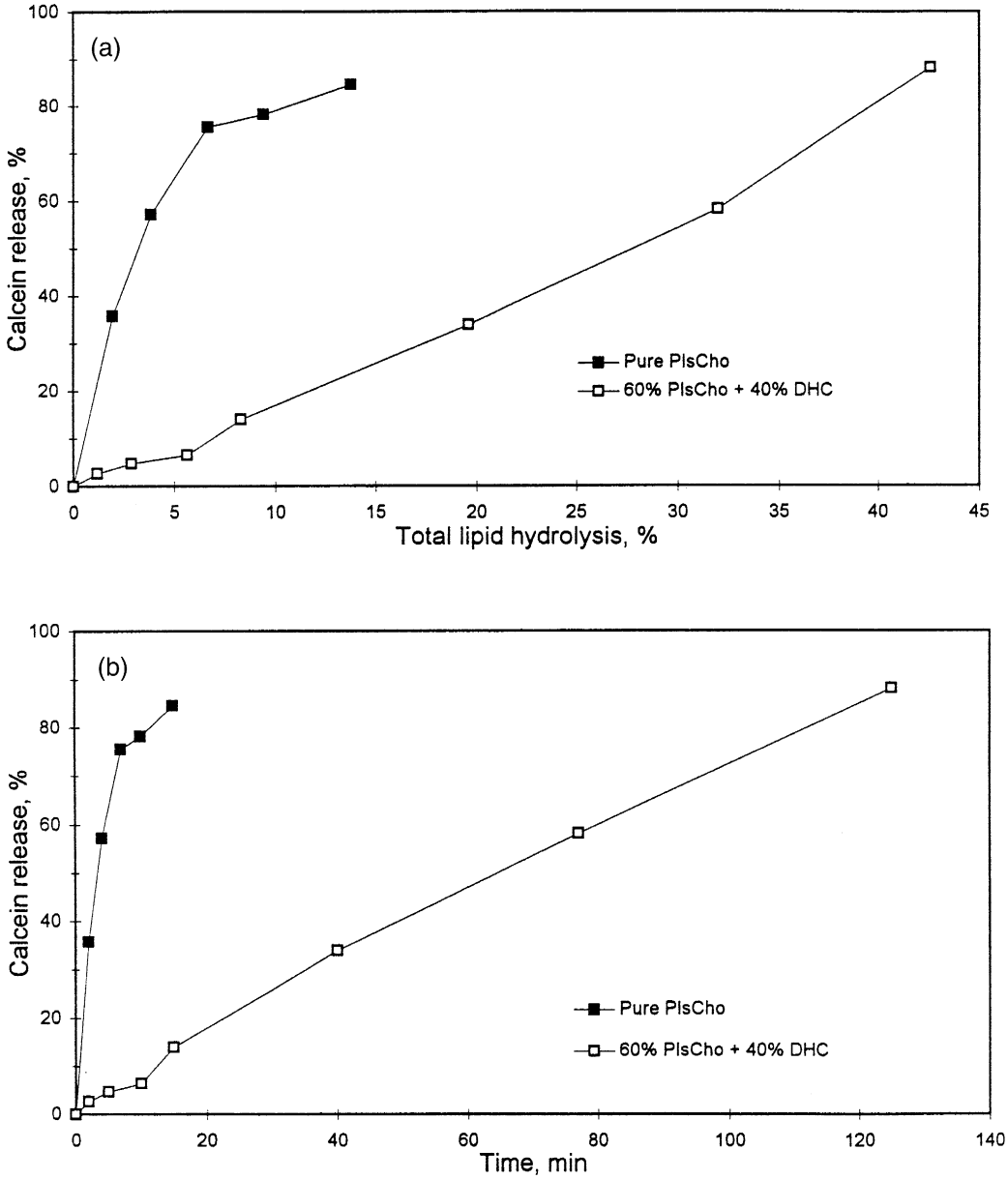


Fig. 5. (a) Calcein release vs. lipid hydrolysis (pH 2.5, 38°C) for liposomes composed of pure PlsPamCho (■) and 6:4 PlsPamCho/DHC (□). (b) Kinetics of calcein release for the same conditions as in (a).

ysis fragments. Equimolar mixtures of fatty acid and lysolipids (the products of diacylphosphatidylcholine hydrolysis) have been shown to initially stabilize [9], and even decrease, the permeability of bilayers containing them up to ≈ 10 mol% overall lipid concentration [41]. These investigators suggested [9,42] that the fatty acid and lysolipid form a 1:1 complex that serves as a structural analog of diradyl glycerophospholipids which do not destabilize the membrane, as would occur with either monomeric hydrolysis product alone. A similar 1:1 complex is less likely to form between fatty aldehydes and lysolipids, since the aldehyde functionality lacks the hydrogen bond-donating capabilities required to stabilize such a complex. In other words, the less polar fatty aldehyde can act only as a hydrogen bond acceptor with the

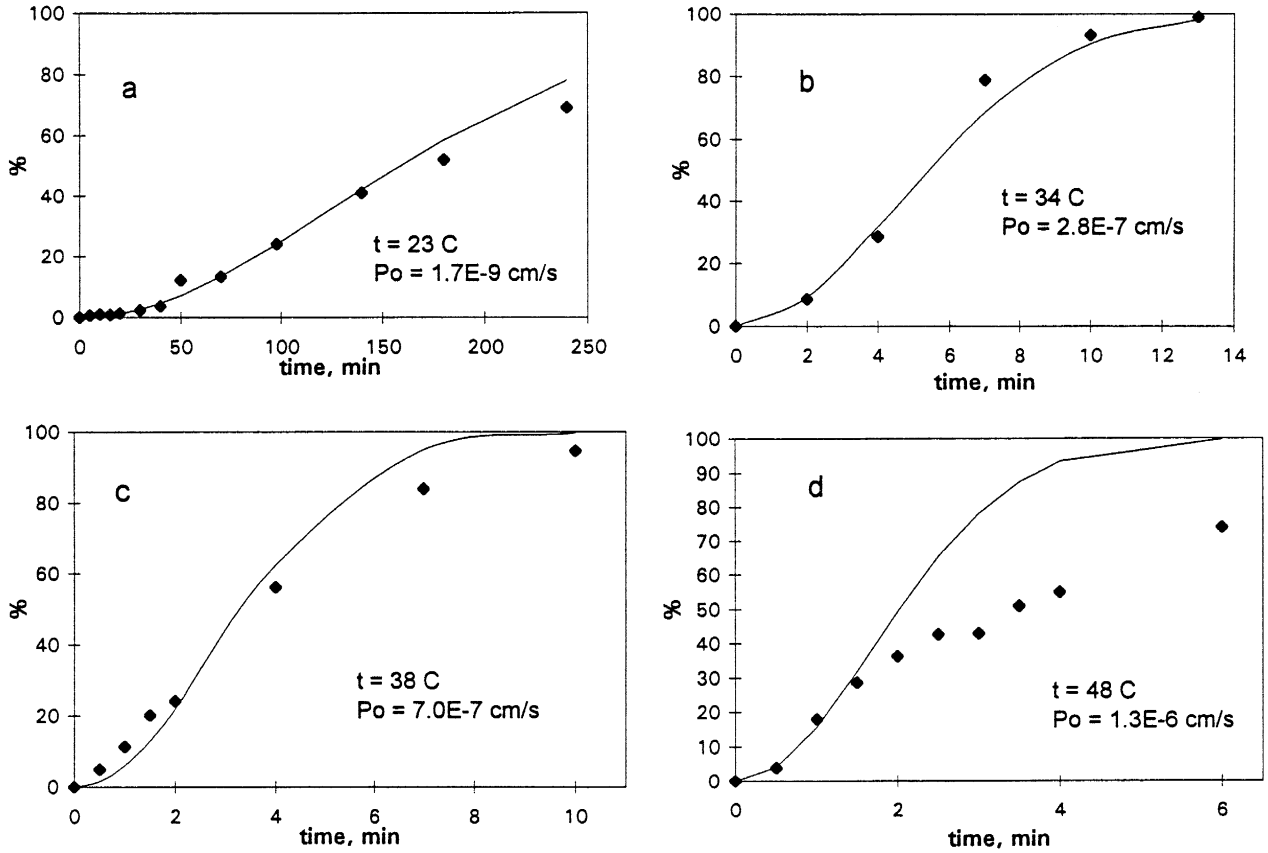


Fig. 6. Temperature dependence of experimental calcein release profiles at pH 2.5 (♦) vs. simulated kinetics (——). (a) $T = 23°C$; (b) $T = 34°C$; (c) $T = 38°C$; (d) $T = 48°C$. P_0 is a proportionality coefficient in the expression $P = P_0 \cdot x$, where P is the PlsPamCho bilayer permeability and x is the degree of lipid hydrolysis. P_0 thus reflects the relative permeability of a plasmenylcholine bilayer containing a given amount of hydrolysis products at different temperatures.

lysolipid co-product to form a single hydrogen bond, whereas the more polar fatty acid is capable of serving as both a hydrogen bond donor and acceptor, thereby forming a pair of hydrogen bonds with a neighboring lysolipid molecule and a stronger 1:1 complex. Plasmenylcholine hydrolysis, therefore, should cause a direct increase in membrane permeability since it produces a net increase in the free lysolipid concentration within the membrane [3]. Increasing fatty aldehyde concentrations may contribute an additional destabilizing effect; however, this has not been demonstrated experimentally. The destabilizing effects caused by the presence of free fatty aldehyde and lysolipid within the membrane bilayer are inhibited by the presence of DHC in PlsPamCho liposomes. Calcein leakage becomes more than an order of magnitude slower when 40% DHC is incorporated within the PlsPamCho bilayer compared to DHC-free liposomes (Fig. 5a and b). The well-documented stabilizing effect that cholesterol incorporation confers upon phospholipid vesicles [4,43,44] appears to occur for PlsPamCho liposomes as well.

3.3.2. Kinetic model for calcein release from hydrolyzed PlsPamCho liposomes

A kinetic model was used to elucidate the leakage mechanism in this liposome system based on the assumption that the bilayer permeability in plasmenylcholine liposomes is directly proportional to its degree of hydrolysis. Using this assumption, the fol-

[3] The role of lysolipid content on vesicle permeability has been reported [41] and is nearly linear within the 0–20% lysolecithin range in vesicles composed of partially hydrogenated egg lecithin mixed with 10 mol% of egg phosphatidylglycerol.

lowing expression was derived (see Appendix A) to fit the kinetic data for calcein leakage:

$$\text{leakage}(\%) = 100\% \cdot \left(1 - \exp\left(\frac{3P_0}{R}\left\{\frac{1}{k} \cdot [-\exp(-kt) - t]\right\}\right)\right)$$

where R is the vesicle radius ($\approx 5 \cdot 10^{-6}$ cm), k is the pseudo-first-order rate constant for plasmenylcholine hydrolysis as determined by HPLC, and 'leakage(%)' is the extent of calcein leakage at a given time t. The coefficient P_0, the only a priori unknown parameter, was found by fitting the calculated kinetics to the experimental points.

Comparisons between the kinetic model and the experimental data are shown in Fig. 6. The model is in reasonably good agreement with the sigmoidal character of calcein release kinetics below T_m (Fig. 6a and b); however, above T_m, the kinetics are slower than predicted by the simple kinetic model (Fig. 6c and d). For example, when the initial phase of the release kinetics was used to fit the experimental points above T_m, the predicted release rates were always faster than the experimentally observed release rates. We believe that this effect is due to partitioning of the plasmenylcholine hydrolysis products between the lipid bilayer and the bulk aqueous phase. Lysolecithin partitioning is known to occur between phosphatidylcholine liposomes and the bulk aqueous phase; at room temperature, the time constant for lysolecithin redistribution between giant liquid crystalline phase EPC liposomes and bulk solution ranges from tens of seconds to minutes, depending on experimental conditions [45]. Since the lysolipid on/off exchange rate should be sensitive to the temperature and phase state of the membrane, it is reasonable to expect that lysolipid partitioning out of the bilayer would be slow below T_m and faster above T_m, such that the actual concentration of hydrolysis products within the bilayer above T_m will be much smaller than expected in the absence of a partitioning process, thereby leading to a slower than expected permeation rate.

3.3.3. Temperature dependence of calcein release rates

The temperature dependencies of calcein release rates (plotted as $1/t_{50\%\ \text{release}}$) and plasmenylcholine hydrolysis rate constants are shown in Fig. 4. These data illustrate that (i) the leakage and lipid hydrolysis rates depend on temperature in a similar way, i.e.,

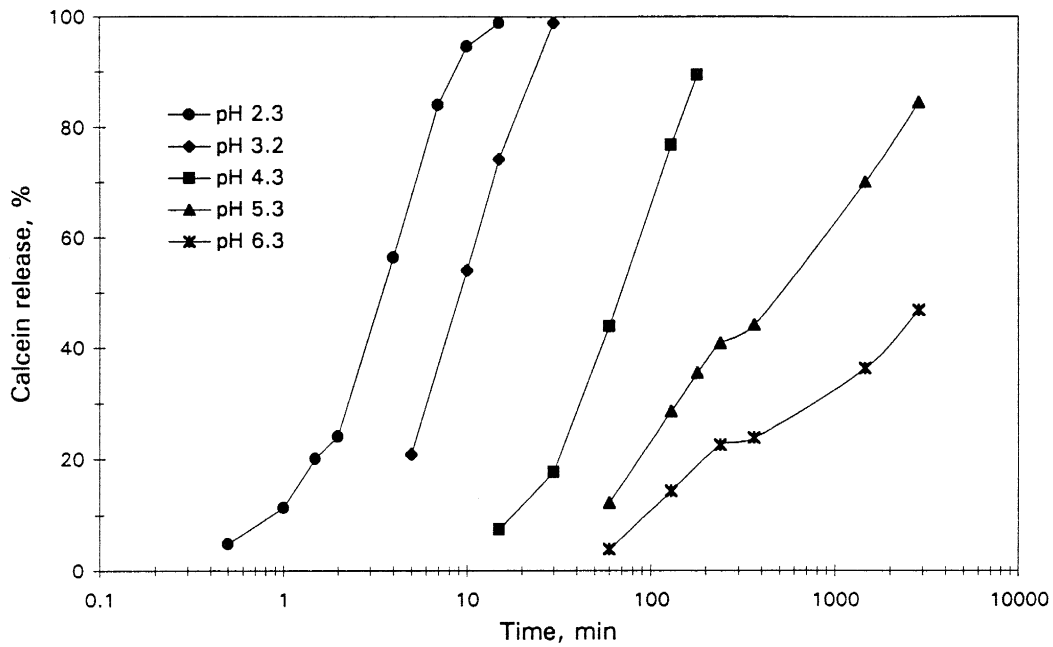


Fig. 7. Effect of pH on the calcein release kinetics from pure PlsPamCho liposomes at 38°C.

below T_m, both rates increase with temperature monotonously until they approach T_m, (ii) in both cases, a plateau or local maximum is observed near T_m (35–42°C), and (iii) below T_m, the liposome leakage rate increases with temperature much faster than the plasmenylcholine hydrolysis rate; above T_m, however, the leakage rates become too fast to be measured using these techniques. Since temperature-dependent permeability increases are known to occur for intact membranes [43], it is reasonable to assume that the membrane permeability for any given degree of hydrolysis will also be temperature-dependent. This explains the much faster increase in leakage rates compared with the more modest increases observed for hydrolysis rates as a function of temperature.

3.3.4. pH dependence

The pH-dependence on calcein efflux from pure PlsPamCho liposomes is shown in Fig. 7. Release rates increase as the solution pH decreases, such that an increase in the proton activity by over four orders of magnitude (from pH 6.3 to 2.5) increases the rate of calcein efflux by approximately three orders of magnitude (i.e., the release rate increases more slowly than the proton activity over this pH range). Contents release from plasmenylcholine liposomes at pH 7.5, however, is quite slow, even in the absence of DHC; control experiments indicate that plasmenylcholine liposomes retain their contents (release $< 10\%$) for more than a week at 38°C and for more than a month at room temperature.

3.3.5. Effect of DHC

Fig. 8 shows the kinetics of calcein release from PlsPamCho liposomes of varying DHC content. Calcein efflux rates remained relatively constant until the liposomal DHC content exceeded 20 mol%; higher DHC contents decreased the observed calcein leakage rates. The rigidifying effect of cholesterol on lipid bilayers is known to decrease the permeability of both lecithin [43,44] and plasmalogen [46] liposomes. In the latter case, the permeability of plasmalogen small unilamellar vesicles for glucose and chloride were reported to decrease by a factor of ≈ 3.5 upon the inclusion of 30% cholesterol in the membrane. A

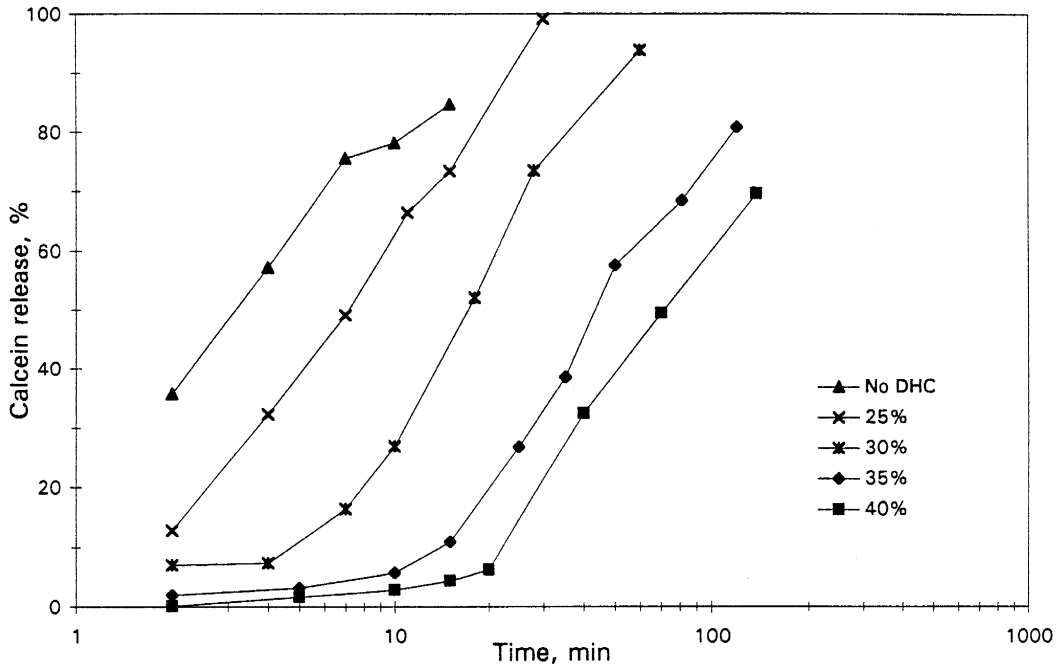


Fig. 8. Effect of DHC on the calcein release kinetics from PlsPamCho/DHC liposomes at 38°C, pH 2.3.

ten-fold change in $t_{50\%\ release}$ occurs for calcein leakage from PlsPamCho liposomes containing 0–40 mol% DHC under acid-catalyzed hydrolysis conditions (Fig. 8). This is in very good agreement with the data reported in [46], considering the differences in leakage mechanism operating for these two systems (i.e., passive leakage across intact membranes vs. triggered release from liposomes undergoing degradation).

3.4. How does plasmenylcholine hydrolysis change bilayer structure and permeability?

Our results show that plasmenylcholine cleavage into lysolipid and fatty aldehyde fragments produces liposomes with increased permeability. A similar effect, arising from lysolipid accumulation within the bilayer, is known to occur during phosphatidylcholine liposome hydrolysis [7,41,47] or upon addition of lysolipid to a preformed liposome solution [2,4–6,43]. Ion channel formation [5], interdigitation of lysolipid in the bilayer [41], pore formation [45], and the formation of discrete defect sites (interlipidic particles or ILP) that are portals for transmembrane transport [7,21], have all been proposed to explain the lysolipid effect on bilayer structure and permeability. The data reported here are more consistent with the formation of membrane defects, since we observe an approximately linear relationship between the degree of hydrolysis and release rates as would be expected if ILP behave like micelles above the CMC (i.e., where the number of ILP defect sites is proportional to the lysolipid concentration). The ILP model is supported by a large number of experimental studies [48–50], and allows a direct analogy between interlipidic particles localized within the bilayer plane and micelles in bulk solution. The pore hypothesis, on the other hand, presumes that lysolipid promotes pore formation in the bilayer by minimizing the pore edge energy, thereby stabilizing the pore edges [45]. The pore hypothesis, however, may also be used to explain our data under the conditions where the total pore area in the liposomal membrane is proportional to the total lysolipid concentration. Under conditions where the membrane pores are small and dynamic, these two concepts (‘ILP’ and ‘pores’) are essentially indistinguishable. Additional studies are under way in this laboratory to further elucidate the mechanistic details of solute permeation through plasmenylcholine bilayers initiated by hydrolytic and photooxidative degradation pathways.

3.5. Conclusions

These data show that the permeability of pure plasmenylcholine liposomes is directly proportional to the extent of vinyl ether hydrolysis. The behavior of plasmenylcholine bilayers during the course of hydrolysis, therefore, differs from that of phosphatidylcholine bilayers, whose permeabilities are known to have a threshold dependence with respect to the degree of hydrolysis. The presence of dihydrocholesterol substantially decreases the leakage rate of pure plasmenylcholine liposomes. Thus, when cholesterol derivatives are present in the plasmenylcholine bilayer, lipid degradation (by either hydrolysis or photooxidation) must occur to a much greater extent to achieve contents releases that are similar to cholesterol-free systems. In practical terms, approximately 3–40% of the PlsPamCho vinyl ether linkage should be decomposed, depending on the cholesterol content in the bilayer, to achieve release rates on a time scale of 10 min. The rate of lysolipid formation increases with temperature; this, however, does not directly translate to increased contents leakage, probably because the partitioning of lysolipids between the bilayer membrane and the bulk phase is also temperature-dependent. Further efforts to develop a quantitative description of how DHC incorporation affects the observed leakage rates from plasmenylcholine liposomes, as well as to apply our understanding of pH-induced release within the endosomal compartment of cells, are in progress.

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Appendix A. Kinetics of solute leakage through plasmenylcholine membranes undergoing degradative cleavage

Solute permeability across membranes is typically described by Fick's First Law:

$$\frac{dn}{dt} = -P \cdot \Delta C \cdot A \quad \text{(A1)}$$

where n is the number of moles of solute, ΔC is the concentration gradient across the membrane, A is the membrane area, and the membrane permeability parameter, P, has dimensions of length/time. In our case, the solute is originally encapsulated inside vesicles with a relatively small entrapped volume (less than 5%). When the solute is released, it is diluted by at least a factor of 20. Therefore, we can assume ΔC to be approximately equal to the solute concentration inside the vesicles, i.e., $C = n/V$. The volume and surface area for spherical vesicles of radius R, is $V = 4/3 \cdot \pi R^3$ and $A = 4\pi R^2$, respectively. Assuming that $P = P_0 \cdot x$ (where x is degree of plasmenylcholine hydrolysis, see text, and P_0 is a proportionality coefficient), the permeability of the hydrolyzing plasmenylcholine membrane should follow pseudo-first order reaction kinetics:

$$P = P_0 \cdot (1 - \exp(-kt)) \quad \text{(A2)}$$

Combining these two expressions gives the following differential equation describing solute leakage from spherical liposomes undergoing degradative cleavage (i.e., hydrolysis or photooxidation):

$$\frac{dn}{dt} = -\frac{3P_0}{R} \cdot (1 - \exp(-kt)) \cdot n \quad \text{(A3)}$$

After integration, the following expression is obtained for the inner liposome solution:

$$\frac{n}{n_0} = \exp\left(\frac{3P_0}{R}\left\{\frac{1}{k} \cdot [1 - \exp(-kt) - t]\right\}\right) \quad \text{(A4)}$$

where n_0 is the initial number of moles of solute inside the liposomes. This may also be expressed in terms of a percent leakage:

$$\text{leakage}(\%) = 100\% \cdot \left(1 - \exp\left(\frac{3P_0}{R}\left\{\frac{1}{k} \cdot [1 - \exp(-kt) - t]\right\}\right)\right) \quad \text{(A5)}$$

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