Decreased Stability in Liposomal Suspensions: Accelerated Loss of *p*-Nitrophenyl Acetate

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Abstract □ The goal of this investigation was to determine the reason for the previously reported increase in the rate of hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol in the presence of positively charged liposomes. When this charge was due to incorporation of stearylamine, the rate of loss increased 5- to 10-fold relative to the control buffers. This rate enhancement was accompanied by formation of *N*-stearylacetamide, an event which was not previously considered. Similar results were obtained with either L-α- or dimyristoyl phosphatidylcholine. When the positive charge on the liposomes was conferred by the cetrimonium ion, however, the acceleration was replaced by a reduction in rate together with the absence of amide formation. Separation of the continuous phases from the liposomes provided media which were kinetically equivalent to the control buffers, indicating that rate enhancement and reduction were both due to the liposomal phases. Increasing the pH produced an increase in ester clearance values due to the stearylamine-containing liposomal phase, which is consistent with the formation of free amine, providing increased aminolysis. Although amide formation was also observed in stearylamine suspensions, the rate of *p*-nitrophenyl acetate loss was much greater in liposomal suspensions. Accelerated loss in the presence of positively charged liposomes is due to the formation of *N*-stearylacetamide by reaction with stearylamine and not to the positive charge, a hypothesis disproved by use of cetrimonium ion containing liposomes.

Liposomes have been investigated as potential drug delivery systems which might control the absorption and distribution of drugs and enrich the supply of drug to particular cells in the body relative to administration of the drug itself.[1-3] Although many studies have reported the entrapment of therapeutic agents in liposomes, few have examined drug stability in vesicular systems.[4-10]

The enhanced loss of *p*-nitrophenyl acetate in the presence of liposomal suspensions has been previously observed.[6-10] The nature of this rate acceleration was variously attributed to (*a*) stabilization of the hydrolysis transition state by the positive charge on the surface of the liposomes,[6] (*b*) site-selective reactions in surfactant vesicles,[7,8] and (*c*) surface reaction due to acetylation of the phosphate groups in egg phosphatidylcholine liposomes.[9]

The present paper reexamines the stability of *p*-nitrophenyl acetate in positively charged liposomal suspensions prepared from L-α-phosphatidylcholine, cholesterol, and either stearylamine or cetrimonium bromide as a function of pH to establish the reason for accelerated loss of the ester. The observed rates in these liposomal suspensions are compared with those in aqueous buffers.

Liposomes are also prepared from synthetic phospholipids to improve their stability.[11] Loss of *p*-nitrophenyl acetate in the presence of dimyristoyl phosphatidylcholine:stearylamine liposomes resulted in kinetic behavior similar to that observed when L-α-phosphatidylcholine:stearylamine was used.

Experimental Section

Preparation of Liposomes with L-α-Phosphatidylcholine—Liposomes were prepared with L-α-phosphatidylcholine (type V-E, from frozen egg yolk; Sigma Chemical Co.), cholesterol (Sigma), and stearylamine (P-L Biochemicals) in a molar ratio of 6.5:1:2, together with tocopherol, by previously described methods.[6] Liposomes with hexadecyltrimethylammonium (cetrimonium) bromide (Aldrich Chemical Co.) to replace stearylamine (in an equivalent molar quantity) were prepared in the same manner.

Preparation of Liposomes with Dimyristoyl Phosphatidylcholine—The method of preparation was similar to that reported,[6] except dimyristoyl phosphatidylcholine (Avanti Polar Lipids Inc.) was used in place of L-α-phosphatidylcholine to prepare the liposomes. However, since the phase transition temperature of dimyristoyl phosphatidylcholine (~26°C) is higher than that of L-α-phosphatidylcholine (0–4°C), hydration of the lipid surface film with aqueous buffer was carried out at 40°C for 10 min. Sonication (model W-375; Heat Systems-Ultrasonics Inc.) was also performed at 40°C, and all other conditions were unchanged.[6]

The size distribution for each of the liposomal suspensions was determined with an Elzone model XY particle size analyzer after diluting 5 μL of the liposomal suspension with 20 mL of the corresponding buffer.

Synthesis and Isolation of *N*-Stearylacetamide—Molar equivalents of stearylamine, acetyl chloride, and triethylamine were allowed to react overnight in chloroform at 40°C. The mixture was washed five times with 15 mL of cold 10% hydrochloric acid to remove the excess bases as hydrochloride salts. The chloroform layer was then washed and dried with anhydrous sodium sulfate. The amide was isolated by preparative TLC (Silica Gel GF, 20 × 20 cm × 1 mm; Analtech Inc.) with chloroform:methanol (9:1) containing a few drops of ammonium hydroxide. The amide band was visualized with iodine vapors on a control plate (R_f = 0.68), and the corresponding silica was removed from the preparative plate and extracted three times with 10–15 mL of chloroform. The combined extracts were dried with anhydrous sodium sulfate and filtered. The filtrate was evaporated under reduced pressure to obtain the amide. An infrared spectrum (Beckman IR Spectrometer 4230) of the sample indicated characteristic peaks for N — H at 3400 cm^{-1}, C — H at ~3000 cm^{-1}, and C = O at 1650 cm^{-1}.

Thin-Layer Chromatography—Qualitative tests for formation of the amide were performed with 2.5 × 10 cm TLC plates (Silica Gel Uniplates, 250 μM; Analtech, Inc.) developed with (*a*) chloroform:methanol (8:1); (*b*) ethyl acetate:methanol (10:1); (*c*) ether, or (*d*) methylene chloride:methanol (8:2). The R_f values in these solvent systems were 0.79, 0.80, 0.57, and 0.75, respectively. Aqueous mixtures were extracted with chloroform, and portions of the organic phase were dried (sodium sulfate) and examined by TLC, with iodine vapors used for visualization.

Analytical Method—The concentrations of *p*-nitrophenyl acetate and *p*-nitrophenol in buffers and liposomal suspensions were monitored spectrophotometrically (Gilford model 250 spectrophotometer) by a published method,[6] with two minor changes to improve the procedure in liposomal suspensions. Quenched samples were chilled in ice to enhance pellet formation and centrifuged (Sorval Superspeed RC-2B centrifuge) at 15,000 rpm for 15 min at 0°C.

Since the UV spectra of *p*-nitrophenyl acetate and *p*-nitrophenol overlap, the absorbance was measured at 271 and 315 nm. Simultaneous equations for the total absorbances at these two wavelengths were used to derive:

$$10^4 C_1 = 11.36\, A_{271} - 2.89\, A_{315} \quad (1)$$

$$10^4 C_2 = 9.98\, A_{271} - 1.41\, A_{315} \quad (2)$$

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from which concentrations of the ester (C_1) and the phenol (C_2) in the final dilutions were calculated. Beers law plots in 0.5 M formic acid containing 80% methanol provided molar extinction coefficients at 271 and 315 nm of 9.13×10^3 and 1.29×10^3 for C_1 and 2.65×10^3 and 10.39×10^3 for C_2. In all kinetic experiments, the mass balance ($C_1 + C_2$), determined as a function of time, accounted for more than 90% of the initial concentration.

Kinetics in Buffers and Liposomal Suspensions and after Removal of Liposomes—The rate of degradation of *p*-nitrophenyl acetate was studied in aqueous buffers (Table I) and in buffered liposomal suspensions (Tables II and IV). The loss of ester was also monitored in the supernatant and filtrate of liposomal suspensions by the following procedure. After the liposomal suspension was chilled in crushed ice for 15 min, it was ultracentrifuged (Beckman L5-50B Ultracentrifuge) at 41,000 rpm ($105,000 \times g$), 4°C for 50 min. The supernatant was centrifuged again. One half of the final supernatant was used without further treatment, and the other half was filtered (Millex-GS 0.22-μM filters; Millipore Corp.). These were used as solvents for the kinetic experiments and referred to as the supernatant and filtered supernatant, respectively (Tables II and IV, conditions 2 and 3). The kinetic experiments in all other systems were performed as previously reported.[6]

Kinetics in Stearylamine Suspensions—The rate of loss of *p*-nitrophenyl acetate in buffers containing stearylamine in suspension was studied at pH 7.0–8.0, 40°C. The procedure for preparing these suspensions was identical to that for the liposomes, except only stearylamine was used.

Table I—Observed First-Order Rate Constants (k_{obs}) for Hydrolysis of 1.08×10^{-3} M *p*-Nitrophenyl Acetate in Buffered Aqueous Solution at 40°C, μ = 0.4.

pH	Buffer Conc., × 10^2 M			$10^3 k_{obs}$, min^{-1}
	Citric Acid	Sodium Citrate	NaCl	
4.11	5.00	5.00	30.0	0.192
4.09	7.50	7.50	25.0	0.262
4.10	10.0	10.0	20.0	0.317
	NaH_2PO_4	Na_2HPO_4	NaCl	
5.80	3.15	0.393	35.7	0.355
5.88	6.30	0.780	31.4	0.525
5.83[a]	9.45	1.15	27.1	0.780
5.90	12.6	1.57	22.7	1.05
6.85	1.75	2.50	30.8	1.65
6.91	3.50	5.00	21.3	2.75
6.88	5.25	7.50	12.3	3.92
7.03	7.00	10.0	3.00	5.35
7.00[a]	5.80	11.4	0.00	6.00
7.53	0.440	3.13	30.2	3.80
7.55	0.880	6.25	20.4	5.60
7.59	1.29	9.40	10.5	7.44
7.53[a]	1.40	10.0	8.60	7.60
7.53	1.75	12.5	0.800	9.50
8.18	0.23	6.00	21.8	9.77
8.09	0.34	9.00	12.7	12.0
8.09[a]	0.45	12.0	3.60	13.6
	Tris H^+	Tris	NaCl	
7.71	4.40	2.00	35.6	5.40
7.72	8.80	4.00	31.2	9.00
7.66	18.6	8.00	21.4	15.0
8.34	2.50	4.00	37.5	16.2
8.35	5.00	8.00	35.0	24.2
8.36	10.0	16.0	30.0	37.1
	$B(OH)_3$	$B(OH)_4^-$	NaCl	
9.10	2.50	2.50	37.5	75.0
9.12	5.00	5.00	35.0	93.0
9.14	10.0	10.0	30.0	126

[a] Composition of control buffers in liposomal studies.

Results

Hydrolysis in Aqueous Buffers—Apparent first-order rate constants (k_{obs}, in min^{-1}) for loss of *p*-nitrophenyl acetate concentration (C_1) were obtained from plots of ln C_1 versus time which were linear for more than two half-lives (Table I). The intercepts (k_i) from plots of k_{obs} versus total buffer concentration (Fig. 1) were employed in the pH–rate profile representing hydrolysis in the absence of buffer (Fig. 2, curve A). The slope of this plot at pH >7 agrees with the theoretical value of unity for a rate that is first-order in

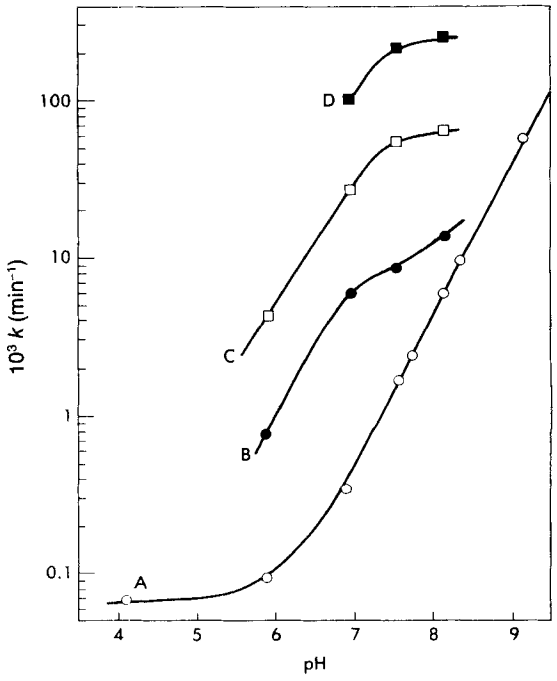


Figure 2—*pH–rate profiles (40°C) for* p-*nitrophenyl acetate in the absence of buffers (k_i) (A) and the control buffers (k_{obs}) (B). Remaining curves are in liposomal suspensions where* C *represents β values (pH 7–8.1) and* k *(pH 5.8), and* D *represents α values (40°C).*

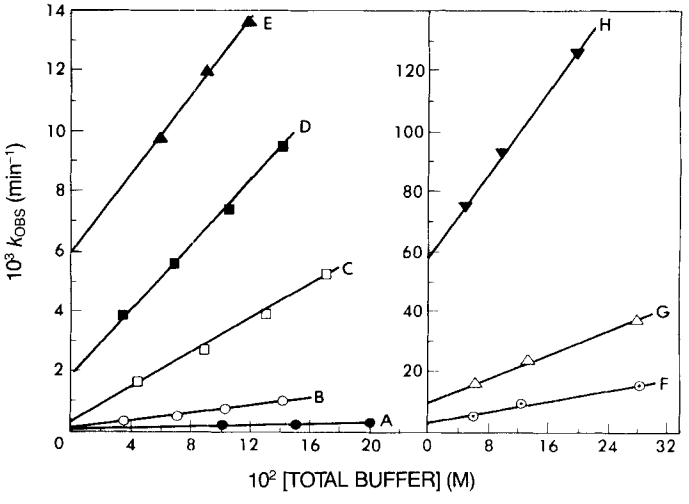


Figure 1—*Observed rate constants for loss of* p-*nitrophenyl acetate at 40°C, μ = 0.4 as a function of total buffer concentration. Citrate:* (A) *pH 4.1. Phosphate:* (B) *pH 5.9;* (C) *pH 6.9;* (D) *pH 7.6;* (E) *pH 8.1. Tris:* (F) *pH 7.7;* (G) *pH 8.4. Borate:* (H) *pH 9.1.*

Table II—Particle Sizes of Stearylamine-Containing Liposomes and Rate Constants for Loss of 1.08×10^{-3} *M* *p*-Nitrophenyl Acetate Described by eq. 5 (f_A, α, f_B, β) or eq. 6 (k) at 40°C

Experimental Conditions[a]	pH	Total Count[b]	Mean Diameter, μM	Percent in 1.5–4.0 μM Range	f_A	f_B	$10^3\alpha$, min^{-1}	$10^3\beta$, min^{-1}	10^3k, min^{-1}
1	8.1	29,900	2.47	82	0.64	0.36	250	65.0	—
1	7.5	34,545	2.51	82	0.48	0.52	220	56.0	—
2	7.5	~800[c]	—	—	0.06	0.94	79.0	7.70	—
3	7.5	~150[c]	—	—	—	—	—	—	7.40
4	7.5	21,789	2.94	81	0.38	0.62	189	22.0	—
1	7.0	28,412	2.50	84	0.38	0.62	101	27.0	—
1	5.8	27,821	2.34	80	—	—	—	—	4.32

[a] Experimental conditions were as follows: (1) liposomal suspensions, (2) supernatant after ultracentrifuging, (3) filtered supernatant, (4) suspensions of dimyristoyl phosphatidylcholine-containing liposomes. See text for details. [b] Count per 200 μL with an Elzone 80XY counter after a 4×10^3-fold sample dilution. [c] Average background count was 100 to 200.

Table III—Clearance Values for Loss of *p*-Nitrophenyl Acetate at 40°C in Suspensions of Stearylamine-Containing Liposomes (CL_{TOTAL}), in Control Buffers (CL_{BUF}), Attributed to the Liposomes (CL_{LIP}), and the Ratios Showing Accelerated Clearance Relative to Controls[a]

pH	CL_{TOTAL}, mL/min	CL_{BUF}, mL/min	CL_{LIP}, mL/min	Clearance Ratios		Rate Constants, Total/Buffer[b]
				CL_{LIP}/CL_{BUF}	CL_{TOTAL}/CL_{BUF}	
5.8[c]	0.0527	0.00952	0.0432	4.5	5.5	5.5
7.0[c]	0.449	0.0761	0.373	4.9	5.9	4.5
7.5[c]	1.06	0.0927	0.967	10.4	11.4	7.4
8.1[c]	1.51	0.166	1.34	8.1	9.1	4.8
7.5[d]	0.404	0.0927	0.311	3.4	4.4	2.9

[a] CL = (initial amount)/(area under the curve) per fixed experimental volume of 12.2 mL. $CL_{TOTAL} = CL_{BUF} + CL_{LIP}$. [b] Rate constant ratios were determined with k/k_B at pH 5.8 and β/k_B at pH ≥7.0. [c] See experimental condition 1 in Table II. [d] See experimental condition 4 in Table II.

Table IV—Particle Sizes of Cetrimonium Ion-Containing Liposomes, Apparent First-Order Rate Constants for Loss of 1.08×10^{-3} *M* *p*-Nitrophenyl Acetate, and the Ratios of These Rate Constants to Those in the Corresponding Buffer Controls (k_B, 40°C, μ = 0.4)

Experimental Conditions[a]	pH	Total Count[b]	Mean Diameter, μM	Percent in 1.5–4.0 μM Range	10^3k, min^{-1}	Ratio, k/k_B
1	7.5	27,504	2.60	78	7.20	0.8
1	7.0	25,423	2.53	75	3.80	0.6
2	7.0	~529[c]	—	—	6.70	1.0
3	7.0	~116[c]	—	—	6.70	1.0

[a] Experimental conditions were as follows: (1) liposomal suspensions, (2), supernatant after ultracentrifuging, (3) filtered supernatant. See text for details. [b] Count per 200 μL determined by using an Elzone model 80XY counter following a 4×10^3-fold sample dilution. [c] Average background count was 100 to 200.

hydroxide. Curve A was drawn by using the equation:

$$k_{obs} = k_s + k_{OH}[OH^-] \quad (3)$$

where $k_s = 6.6 \times 10^{-5}$ min^{-1}, $k_{OH} = 1.51 \times 10^3$ M^{-1} min^{-1}, and $[OH^-] = (2.92 \times 10^{-14})/[H^+]$.

Rate constants in phosphate buffers were described by:

$$k_{obs} = k_s + k_{OH}[OH^-] + k_{HPO_4}[HPO_4^=] \quad (4)$$

where $k_{HPO_4} = 0.055$ M^{-1} min^{-1} and the contribution from $H_2PO_4^-$ is negligible.

Kinetics in Stearylamine-Containing Liposomal Suspensions—The loss of *p*-nitrophenyl acetate in L-α-phosphatidylcholine:stearylamine liposomal suspensions in the pH range 7.0–8.1 was described by:

$$F = f_A e^{-\alpha t} + f_B e^{-\beta t} \quad (5)$$

where F is the fraction remaining at time t and f_A and f_B are the fractions lost in accordance with the rapid (α) and slow (β) rate constants, respectively. The values for f_A, α, f_B, and β were obtained by nonlinear regression (Table II). The mean ± SD of the β values (in min^{-1}) in nine trials at pH 7.5 were 0.057 (0.0087). At pH 5.8, loss was described by:

$$F = e^{-kt} \quad (6)$$

As shown in curves C and D of Fig. 2, the rate constants in the presence of stearylamine-containing liposomes were larger than those in the corresponding control buffers (curve B).

The loss of *p*-nitrophenyl acetate in the supernatant of an ultracentrifuged liposomal suspension (pH 7.5) was described by eq. 5, where the β value is approximately equal to buffer control, k_B. However, the contribution of $f_A e^{-\alpha t}$ to the total area under the curve (AUC) is less than 5%, which may reflect a few remaining liposomes since the residual total count is slightly higher than background. After filtration of this supernatant, the count returned to background, and the first-order rate constant (eq. 6) was equal to the control (Table II).

Accelerated loss in liposomal suspensions can be assessed by calculating the total clearance values from the AUC from zero to infinity, where:

$$CL_{TOTAL} = \text{(initial amount)/AUC} \quad (7)$$

for the fixed volume (12.2 mL) used throughout this study. Rate of ester loss using clearance values allows comparisons of biexponential loss in liposomal suspensions (eq. 5) to monoexponential loss (eq. 6) in control buffers (Table III). This also provides a means for calculating clearance values due to the liposomal phase (CL_{LIP}). Since clearance is additive and the continuous phase is kinetically equivalent to buffer controls, the following relationship holds:

$$CL_{TOTAL} = CL_{BUF} + CL_{LIP} \quad (8)$$

where CL_{BUF} represents nonliposomal clearance calculated from the buffer controls.

In liposomal suspensions containing dimyristoyl phosphatidylcholine in place of L-α-phosphatidylcholine, loss was described by eq. 5 (Table II, condition 4). Clearance values again indicated rate enhancement (Table III).

Kinetics in Cetrimonium Ion Containing Liposomal Suspensions—When the positive charge in the L-α-phosphatidylcholine liposomes was conferred by the cetrimonium ion in place of stearylamine, the loss of ester was first-order, with a slight reduction in rate relative to the controls (Table IV). Both the supernatant following ultracentrifuging and the filtered supernatant provided first-order rate constants that were equal to those in the control buffers (Table IV, conditions 2 and 3).

Discussion

It was previously proposed that the increased loss of *p*-nitrophenyl acetate was due to the positively charged surface of the liposomes favoring formation of the negatively charged transition state during nucleophilic hydroxide-ion attack.[6] Results now indicate that liposomal stearylamine reacts with *p*-nitrophenyl acetate to form *N*-stearylacetamide, thus increasing the rate of loss of the ester. The salient observations supporting this conclusion are summarized in Table V and discussed individually below.

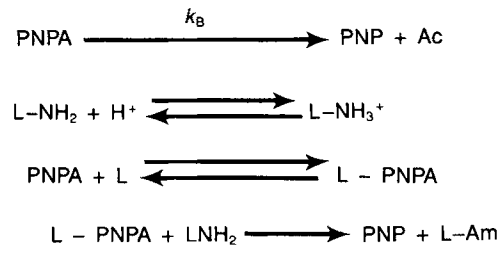
In the presence of stearylamine-containing liposomes, the loss of *p*-nitrophenyl acetate is ~5–10 times faster than the buffer controls. This accelerated loss is accompanied by the formation of *N*-stearylacetamide. Removal of these liposomes results in a rate equal to the control with no amide formation. Therefore, if stearylamine is present in the aqueous phase as either uncountable monomers or micelles, it does not contribute measurably to the observed rate enhancement.

Replacing L-α-phosphatidylcholine with dimyristoyl phosphatidylcholine resulted in a 4.4-fold increase in ester loss accompanied by formation of the amide. The smaller rate enhancement relative to the L-α-phosphatidylcholine might be due to the reduction in total liposome count and the change in the environment surrounding the stearylamine within the liposomes.

Rate constants in buffered suspensions of stearylamine at concentrations similar to those used in liposomes were larger than controls at pH 8.4, at which tests were positive for *N*-stearylacetamide. At pH 6.9, the absence of both amide formation and rate enhancement suggest the lack of unprotonated amine to act as the nucleophile.

Suspensions of cetrimonium ion containing liposomes provided first-order loss of ester with a 30% reduction in rate relative to control buffers, together with negative tests for amide formation. If it is assumed that the positive charge is not catalytic, then the 30% reduction is similar to that reported for *p*-nitrophenyl acetate loss in stearylamine-free liposomes of neutral and negative charges.[6] The rate of ester loss in the aqueous phase, which was separated from the cetrimonium ion containing liposomes, was equal to that in the control buffer. Cetrimonium ion in the continuous phase of the suspension is therefore not a significant influence on the observed rate constant.

Loss of ester in the presence of stearylamine-containing liposomes in suspension involves the processes in Scheme I. Stearylamine in the liposomes (L-NH_2) behaves as a nucleophile which effectively competes with hydrolysis of *p*-nitrophenyl acetate (PNPA) by forming the alternate product, *N*-stearylacetamide (L-Am). Formation of *p*-nitrophenol (PNP) is common to both reactions, which explains the mass balance observed when the ester and phenol concentrations were summed as a function of time.



Scheme I

Association of *p*-nitrophenyl acetate with the liposomes (L-PNPA) can result in aminolysis when the proximity of free amine (L-NH_2) is favorable for the reaction. The increased reactivity of stearylamine in liposomal suspensions may be due to the combination of increased effective concentration, increased basicity, and increased reactivity due to proximity of reactants. Potentiometric titrations of stearylamine-containing liposomal suspensions provided apparent pK_a values in the range of 7 to 8. The low aqueous solubility of stearylamine itself precluded its titration, but the expected pK_a value for this aliphatic amine would be close to 10. The apparent decrease in basicity of the liposome surface over that expected for an aliphatic amine is consistent with changes observed for other acids and bases incorporated into liposomes.[12] There is also the possibility for rate enhancement due to more favorable intermolecular relationships between bound ester (L-PNPA) and neighboring free amine (L-NH_2). Reactants which are held in relatively fixed and favorable orientations would undergo enhanced aminolysis in comparison with the relatively mobile reactants in a solution.

Although it is also possible that hydrolysis of ester could occur in the liposome-associated phase (L-PNPA), this is not likely to be a significant pathway, since stearylamine-free liposomes are known to reduce the hydrolysis rate.[6] The

Table V—Summary of Ratios of Total Clearance Values[a] for the Loss of *p*-Nitrophenyl Acetate in Various Experimental Systems[b] Relative to Values in the Corresponding Buffer Controls (40°C) and Test Results for *N*-Stearylacetamide

Experimental Conditions	pH	10^{-5} Count per μL, Mean ± SD	Clearance Relative to Control	Amide Test
Stearylamine Liposomes	5.8–8.1	6.0 ± 0.6	8.0 ± 2.8	+
After Liposome Removal	7.5	Background	1.0	–
Stearylamine–DMPC[c] Liposomes	7.5	4.5	4.4	+
Stearylamine Suspensions	6.9		1.0	–
	8.4		1.4	+
Cetrimonium Ion-Containing Liposomes	7.0–7.5	5.3	0.7	–
After Liposome Removal	7.0	Background	1.0	–

[a] Clearance = (initial amount)/(area under the curve) per 12.2 mL. For monoexponential loss, the ratios of the rate constants equal the clearance ratios. [b] See text for details. [c] Abbreviation: DMPC, dimyristoyl phosphatidylcholine (used in place of L-α-phosphatidylcholine).

estimated contribution of general-base catalysis by liposomal stearylamine, based on a Bronsted plot with the buffer catalytic constants, is negligible relative to the observed rate of ester loss in the presence of liposomes.

Finally, amide formation accompanied each case in which accelerated loss was observed. Decreasing the pH resulted in decreased clearance values for stearylamine-containing liposomes (Table III, CL_{LIP}). This is consistent with a reduction in free amine concentration due to protonation, which would also increase the positive charge on the liposomes. Furthermore, a rate reduction results when the positive charge is due to cetrimonium ion. The ester instability is therefore not related to the liposomal charge but is due instead to aminolysis, which competes preferentially with hydrolysis.

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