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Pejaver, Satish K.

KINETICS IN LIPOSOMAL SYSTEMS. DRUG STABILIZATION; SYNTHESIS AND DEGRADATION OF LIPOSOME PRODRUGS

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KINETICS IN LIPOSOMAL SYSTEMS. DRUG STABILIZATION;
SYNTHESIS AND DEGRADATION OF LIPOSOME PRODRUGS

DISSertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Satish K. Pejaver, B. Pharm.

* * * * *

The Ohio State University
1986

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Robert E. Notari, Adviser
College of Pharmacy
To my parents
ACKNOWLEDGEMENTS

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Thanks are also due to the Pharmaceutics faculty, staff and fellow graduate students from whom I have learned much and enjoyed their friendship.

I would like to specially thank my grandmother, parents and sister for their undevoted love, understanding and consistent encouragement throughout my studies.
VITA

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Decreased Stability in Liposomal Suspensions: Accelerated Loss of
1985, 74, 1167.

FIELD OF STUDY

Major Field: Pharmaceutics and Pharmaceutical Chemistry
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ORGANIZATION OF THESIS

This dissertation is divided into three chapters each containing an introduction with detailed literature search.

In a previous report accelerated loss of p-nitrophenyl acetate was attributed to positively charged liposomes in suspension. In Chapter I, a reexamination of this phenomenon proves that this acceleration is due to formation of N-stearylacetamide via nucleophilic attack on the ester by the stearylamine in the liposomes.

It was hypothesized that stable drug solutions encapsulated in liposomes would remain protected subsequent to dispersion of the vesicles in an unfavorable environment where the drug is known to be unstable. In Chapter II, stabilization of ancitabine was achieved using this approach. Liposomes offered significant protection only when the phase transition temperature of the phospholipid in the liposome was greater than the storage temperature of the suspension.

Chapter III examines the possibility that a drug may be bioreversibly bonded to functional groups on the surface of the liposomes thus forming a potential liposome prodrug. Success was realized in the attachment of a model compound, p-nitrophenol, to the stearic acid present in the liposomal wall. The storage stability and the enzymatic conversion of this liposome prodrug ester were examined.
CHAPTER I

DECREASED STABILITY IN LIPOSOMAL SUSPENSIONS:

ACCELERATED LOSS OF P-NITROPHENYL ACETATE*

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SUMMARY

The goal of this investigation was to determine the reason for the previously reported increase in rate of loss of p-nitrophenyl acetate 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 which was not previously considered. Replacement of L-α-phosphatidylcholine with dimyr-istoyl phosphatidylcholine did not markedly alter the results. However, when the positive charge on the liposomes was conferred by the quaternary amine, cetrimonium, 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 formation of free amine providing increased aminolysis. While 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 formation of N-stearylacetamide by reaction with stearylamine and not due to the positive charge, a hypothesis disproved using cetrimonium-containing liposomes.
INTRODUCTION

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.\textsuperscript{1-3} Although many studies report on entrapment of therapeutic agents in liposomes, few have examined drug stability in vesicular systems.\textsuperscript{4-10}

The enhanced loss of p-nitrophenyl acetate has been previously observed.\textsuperscript{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\textsuperscript{6} (b) site selective reactions in surfactant vesicles\textsuperscript{7,8} and (c) surface reaction due to acetylation of the phosphate groups in egg phosphatidylcholine liposomes.\textsuperscript{9}

The present paper re-examines the stability of p-nitrophenyl acetate in positively-charged liposomal suspensions prepared from L-\textalpha-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 to those in aqueous buffers.
Liposomes are also prepared from synthetic phospholipids to improve their stability. Studies using dimyristoyl phosphatidylcholine:stearylamine liposomes resulted in similar kinetic behavior for p-nitrophenyl acetate loss.
EXPERIMENTAL

Preparation of Liposomes with L-α-phosphatidylcholine: --Sixteen millilitres of liposomal suspension was prepared with 10 mg L-α-phosphatidylcholine, 7.4 mg cholesterol (Sigma Chemical Corp.), and 10.4 mg stearylamine (P-L Biochemicals) in the molar ratio of 6.5:1:2 together with α-tocopherol by previously described methods. Liposomes using 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 except that 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, while all other conditions were unchanged.
The size distribution for each of the liposomal suspensions was determined using an Elzone Model XY particle size analyzer after diluting 5 μL of the liposomal suspension with 20 mL of the corresponding buffer. A typical size distribution curve is shown in Fig. 1.1.

**Synthesis and Isolation of N-Stearylacetamide:** Molar equivalents of stearylamine, acetylchloride and triethylamine were reacted 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 using anhydrous sodium sulphate. The amide was isolated by preparative thin layer chromatography (Silica Gel GF, 20 x 20 cm, 1 mm, Analtech Inc.) using chloroform:methanol (9:1) containing a few drops of ammonium hydroxide. The amide band was visualized using iodine vapors on a control plate ($R_f = 0.68$) and the corresponding silica removed from the preparative plate and extracted three times with 10-15 mL chloroform. The combined extracts were dried with anhydrous sodium sulfate and filtered. The filtrate was evaporated to dryness 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 \(\sim\)3000 cm\(^{-1}\) and C=O at 1650 cm\(^{-1}\).

**Thin Layer Chromatography:** Qualitative tests for formation of the amide were performed using 2.5 x 10 cm TLC plates (Silica Gel Unip-
lates, 250 M, Analtech, Inc.) developed with (a) chloroform:methanol (8:1); (b) ethylacetate: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 reaction mixtures were extracted with chloroform and aliquots of the organic phase were dried (sodium sulfate) and examined by TLC using iodine vapors for visualization.

**Analytical:** --The concentrations of p-nitrophenyl acetate and p-nitrophenol in buffers and liposomal suspensions were monitored spectrophotometrically (Gilford, Model 250, Spectrophotometer) using a published method 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, (Fig. 1.2) the absorbance was measured at 271 and 315 nm. Simultaneous equations for the total absorbances at these two wavelengths were used to derive:

$$10^5 C_1 = 11.36 A_{271} - 2.89 A_{315}$$

(1.1)

$$10^5 C_2 = 9.98 A_{315} - 1.41 A_{271}$$

(1.2)

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 $9.13 \times 10^3$ and $1.29 \times 10^3$ for $C_1$ and $2.65 \times 10^3$
and $10.39 \times 10^3$ for $C_2$ (Figs. 1.3, 1.4).

In all kinetic experiments, the mass balance ($C_1$ plus $C_2$), deter-
minded as a function of time, accounted for more than 90% of the initial
concentration.

**Kinetics in Buffers, Liposomal Suspensions and After Removal of
Liposomes:** --The rate of degradation of p-nitrophenyl acetate was
studied in aqueous buffers (Table 1.1) and in buffered liposomal sus-
pensions (Tables 1.2 and 1.4). The loss of ester was also monitored
in the supernatant and filtrate of liposomal suspensions by the follow-
ing procedure. After chilling the liposomal suspension in crushed ice
for 15 min, it was ultracentrifuged (Beckman L5-50B Ultracentrifuge)
at 41,000 rpm (105,000 g), 4°C for 50 min. The supernatant was cen-
trifuged again. One-half of the final supernatant was used without
further treatment and the other half was filtered (Millipore GS 0.22 μM
Filters, Millipore Corp.). These were used as solvents for the kinetic
experiments and referred to as the supernatant and filtered superna-
tant respectively (Tables 1.2 and 1.4, 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-nitrophenylacetate in buffers containing stearylamine in suspension
was studied at pH 7.0-8.0, 40°C (Table 1.5). The procedure for pre-
paring these suspensions was identical to that for the liposomes except that only stearylamine was used.
RESULTS

Hydrolysis in Aqueous Buffers: -- Apparent first-order rate constants \( k_{\text{obs}} \) in \( \text{min}^{-1} \) for loss of p-nitrophenol acetate concentration \( (C_1) \) were obtained from plots of \( \ln C_1 \) versus time (Fig. 1.6) which were linear for more than two half-lives (Table 1.1). The intercepts \( (k_i) \) from plots of \( k_{\text{obs}} \) versus total buffer concentration (Fig. 1.5) were employed in the pH-rate profile representing hydrolysis in the absence of buffer (Fig. 1.7, curve A). The slope of this plot at \( \text{pH} > 7 \) agrees with the theoretical value of unity for a rate that is first order in hydroxide. Curve A was drawn using the equation

\[
    k_{\text{obs}} = k_s + k_{\text{OH}} \cdot \text{OH}^- \tag{1.3}
\]

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

Rate constants in phosphate buffers were described by

\[
    k_{\text{obs}} = k_s + k_{\text{OH}} \cdot \text{OH}^- + k_{\text{HPO}} \cdot \text{HPO}_4^- \tag{1.4}
\]

where \( k_{\text{HPO}} = 0.055 \text{ M}^{-1} \text{ min}^{-1} \) and the contribution from \( \text{H}_2\text{PO}_4^- \) 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 to 8.1 was described by

\[ F = f_A e^{-αt} + f_B e^{-βt} \]  
\[ (1.5) \]

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 1.2). The mean and standard deviation of the \( β \) values (min\(^{-1}\)) in nine trials at pH 7.5 were 0.057 (0.0087). At pH = 5.8, loss was described by

\[ F = e^{-kt} \]  
\[ (1.6) \]

As shown in curves C and D of Fig. 1.7, the rate constants in the presence of stearylamine-containing liposomes are 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. 1.5 where the \( β \) value is approximately equal to buffer control, \( k_B \). However, the contribution of \( f_A e^{-αt} \) 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. 1.6) was equal to the control (Table 1.2).
Accelerated loss in liposomal suspensions can be assessed by calculating the total clearance values,

$$\text{CL}_{\text{TOTAL}} = \frac{\text{Initial Amount}}{\text{AUC}}$$  \hspace{1cm} (1.7)

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. 1.5) to monoexponential loss (Eq. 1.6) in control buffers (Table 1.3). This also provides a means for calculating clearance values due to the liposomal phase (CL\text{LIP}). Since clearance is additive and the continuous phase is kinetically equivalent to buffer controls the following relationship holds

$$\text{CL}_{\text{TOTAL}} = \text{CL}_{\text{BUF}} + \text{CL}_{\text{LIP}}$$ \hspace{1cm} (1.8)

where $\text{CL}_{\text{BUF}}$ represents non-liposomal clearance calculated from the buffer controls.

In liposomal suspensions using dimyristoyl phosphatidylcholine in place of $\text{L-}\alpha\text{-phosphatidylcholine}$, loss was described by Eq. 1.5 (Table 1.2, condition 4). Clearance values again indicate rate enhancement (Table 1.3).

**Kinetics in Cetrimonium-Containing Liposomal Suspensions:** When the positive charge in the $\text{L-}\alpha\text{-phosphatidylcholine}$ liposomes was conferred by the quaternary amine, cetrimonium, in place of stearylamine, the loss of ester was first order with a slight reduction in rate relative to...
the controls (Table 1.4, Fig. 1.8). Both the supernatant following ultracentrifuging and the filtered supernatant provided first-order rate constants that were equal to those in the control buffers (Table 1.4, conditions 2 and 3).
DISCUSSION AND CONCLUSIONS

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. 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 1.6 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, either as non-countable monomers or micelles, it does not contribute measurably to the observed rate enhancement.

Replacing L-a-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-a-phosphatidylcholine, may 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 where tests were positive for N-stearylacamide. 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-containing liposomes provided first order loss of ester with a 30% reduction in rate relative to control buffers (Fig. 1.8) together with negative tests for amide formation. Assuming 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 charge. The rate of ester loss in the aqueous phase, which was separated from the cetrimonium-containing liposomes, was equal to that in the control buffer. This rules out cetrimonium in the continuous phase of the suspension as a potential influence on the observed rate constant.

Loss of ester in the presence of stearylamine-containing liposomes in suspension involves the processes in Scheme 1.1. Stearylamine in the liposomes (L-NH₂) behaves as a nucleophile which effectively competes with hydrolysis of p-nitrophenyl acetate (PNPA) by forming the alternate product, N-stearylacamide (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.

(1) PNPA $\xrightarrow{k_B} \text{PNP} + \text{Ac}$
(2) $\text{LNH}_2 + \text{H}^+ \leftrightarrow \text{LNH}_3^+$
(3) PNPA + L $\leftrightarrow$ L - PNPA
(4) L - PNPA + $\text{LNH}_2 \rightarrow \text{PNP} + \text{L-Am}$

Scheme 1.1

While the proposed scheme cannot be solved for the individual rate constants using the available data the following procedure demonstrates that it behaves in a similar fashion to that observed experimentally. The assay, applied to the liposomal suspensions, measured the total ester present in the aqueous phase (PNPA) and associated with the liposomes (L-PNPA). The time course for total ester concentration was described using a biexponential equation at pH 7 to 8 (Eq. 1.5) which collapsed to a monoexponential equation at pH 6 (Eq. 1.6) with an observed increase in rate relative to buffer controls in all cases. The time courses for total ester concentration (PNPA + L-PNPA) were simulated by computer using the Runge Kutta algorithm for the differential equations associated with Scheme 1.1. The required parameter values were arbitrarily assigned since they are not intended to repre-
sent actual values for the various constants. The intent was to demonstrate that Scheme 1.1 was capable of the observed behavior for the experimental situations.

Figure 1.9 illustrates the agreement between the computer simulation and the experimental data at pH 7.53. Figure 1.9 also illustrates the simulated appearance of amide which reflects the loss of ester by process 4 and the appearance of acetate which is indicative of process 1. In these simulations the contribution of hydrolysis (process 1) to the overall degradation of the ester was negligible. This is consistent with the suggestion that acceleration is due primarily to aminolysis.

Association of p-nitrophenyl acetate with the liposomes (L-PNPA) can result in aminolysis when the proximity of free amine (LNH$_2$) 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 pKa values in the range of 7 to 8 (Fig. 1.10). The low aqueous solubility of stearylamine itself precluded its titration, but the expected pKa value for this aliphatic amine would be close to 10. The apparent increase 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.$^{13}$ 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$_2$). Those reactants which are held in relatively fixed and favorable orientations would undergo enhanced aminolysis in comparison to the relatively mobile reactants in a solution.

While 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. The estimated contribution of general-base catalysis by liposomal stearylamine based on a Bronsted plot (Fig. 1.11) using 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 wherein accelerated loss was observed. Decreasing the pH resulted in decreased clearance values for stearylamine-containing liposomes (Table 1.3, $C_{LIP}$). 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. Thus the ester instability is not related to the liposomal charge but is due instead to aminolysis which competes preferentially with hydrolysis.
REFERENCES

3. Ryman, B.E. "The use of liposomes as carriers of drugs and other cell modifying molecules"; Proceedings of the Sixth International Congress of Pharmacology; Vol. 5, Helsinki, Finland, 1975, pp. 91-103.


<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer Concentration</th>
<th>$10^3 k_{obs}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citric Acid</td>
<td>Sodium Citrate</td>
</tr>
<tr>
<td>4.11</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>4.09</td>
<td>7.50</td>
<td>7.50</td>
</tr>
<tr>
<td>4.10</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>5.80</td>
<td>3.15</td>
<td>0.393</td>
</tr>
<tr>
<td>5.88</td>
<td>6.30</td>
<td>0.780</td>
</tr>
<tr>
<td>5.83a</td>
<td>9.45</td>
<td>1.15</td>
</tr>
<tr>
<td>5.90</td>
<td>12.6</td>
<td>1.57</td>
</tr>
<tr>
<td>6.85</td>
<td>1.75</td>
<td>2.50</td>
</tr>
<tr>
<td>6.91</td>
<td>3.50</td>
<td>5.00</td>
</tr>
<tr>
<td>6.88</td>
<td>5.25</td>
<td>7.50</td>
</tr>
<tr>
<td>7.03</td>
<td>7.00</td>
<td>10.0</td>
</tr>
<tr>
<td>7.00a</td>
<td>5.80</td>
<td>11.4</td>
</tr>
<tr>
<td>7.53</td>
<td>0.440</td>
<td>3.13</td>
</tr>
<tr>
<td>7.55</td>
<td>0.880</td>
<td>6.25</td>
</tr>
<tr>
<td>7.59</td>
<td>1.29</td>
<td>9.40</td>
</tr>
<tr>
<td>7.53a</td>
<td>1.40</td>
<td>10.0</td>
</tr>
<tr>
<td>7.53</td>
<td>1.75</td>
<td>12.5</td>
</tr>
<tr>
<td>8.18</td>
<td>0.23</td>
<td>6.00</td>
</tr>
<tr>
<td>8.09</td>
<td>0.34</td>
<td>9.00</td>
</tr>
<tr>
<td>8.09</td>
<td>0.45</td>
<td>12.0</td>
</tr>
</tbody>
</table>

|  | TRISH$^+$ | TRIS |
|  | 4.40 | 2.00  | 35.6  | 5.40  |
|  | 8.80 | 4.00  | 31.2  | 9.00  |
|  | 18.6 | 8.00  | 21.4  | 15.0  |
|  | 2.50 | 4.00  | 37.5  | 16.2  |
|  | 5.00 | 8.00  | 35.0  | 24.2  |
|  | 10.0 | 16.0  | 30.0  | 37.1  |

|  | B(OH)$_3$ | B(OH)$_4^-$ |
|  | 2.50 | 2.50  | 37.5  | 75.0  |
|  | 5.00 | 5.00  | 35.0  | 93.0  |
|  | 10.0 | 10.0  | 30.0  | 126.0 |

a Composition of control buffers in liposomal studies.
Table 1.2 — Particle Sizes of Stearylamine-containing Liposomes and Rate Constants for Loss of 1.08 x 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\textsuperscript{a}: (1) Liposomal Suspensions, (2) Supernatant After Ultracentrifuging, (3) Filtered Supernatant, and (4) Suspensions of Dimyristoyl Phosphatidylcholine-containing Liposomes.

<table>
<thead>
<tr>
<th>Exptl. Condition</th>
<th>pH</th>
<th>Total count \textsuperscript{b}</th>
<th>Mean Diameter</th>
<th>% in 1.5-4.0 μM range</th>
<th>f_A</th>
<th>f_B</th>
<th>10\textsuperscript{3}α (min\textsuperscript{-1})</th>
<th>10\textsuperscript{3}β (min\textsuperscript{-1})</th>
<th>10\textsuperscript{3}k (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.1</td>
<td>29,900</td>
<td>2.47</td>
<td>82</td>
<td>0.64</td>
<td>0.36</td>
<td>250</td>
<td>65.0</td>
<td>----</td>
</tr>
<tr>
<td>1</td>
<td>7.5</td>
<td>34,545</td>
<td>2.51</td>
<td>82</td>
<td>0.48</td>
<td>0.52</td>
<td>220</td>
<td>56.0</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>7.5</td>
<td>~800\textsuperscript{c}</td>
<td>----</td>
<td>----</td>
<td>0.06</td>
<td>0.94</td>
<td>79.0</td>
<td>7.70</td>
<td>----</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>~150\textsuperscript{c}</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>7.40</td>
</tr>
<tr>
<td>4</td>
<td>7.5</td>
<td>21,789</td>
<td>2.94</td>
<td>81</td>
<td>0.38</td>
<td>0.62</td>
<td>189</td>
<td>22.0</td>
<td>----</td>
</tr>
<tr>
<td>1</td>
<td>7.0</td>
<td>28,412</td>
<td>2.50</td>
<td>84</td>
<td>0.38</td>
<td>0.62</td>
<td>101</td>
<td>27.0</td>
<td>----</td>
</tr>
<tr>
<td>1</td>
<td>5.8</td>
<td>27,821</td>
<td>2.34</td>
<td>80</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>4.32</td>
</tr>
</tbody>
</table>

\textsuperscript{a}See text for details.

\textsuperscript{b}Count per 200 μL using an Elzone 80XY counter following a 4 x 10\textsuperscript{3} fold sample dilution.

\textsuperscript{c}Average background count = 100 to 200.
Table 1.3 Clearance Values\(^a\) (in mL/min) 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. Rate Constant Ratios Use k/k\(_B\) at pH 5.8 and \(\beta/k_B\) at pH ≥ 7.0.

<table>
<thead>
<tr>
<th>pH</th>
<th>CL(_{TOTAL})</th>
<th>CL(_{BUF})</th>
<th>CL(_{LIP})</th>
<th>CLEARANCE RATIOS (LIP/BUF)</th>
<th>RATE CONSTANTS (TOTAL/BUF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8(^b)</td>
<td>0.0527</td>
<td>0.00952</td>
<td>0.0432</td>
<td>4.5</td>
<td>5.5</td>
</tr>
<tr>
<td>7.0(^b)</td>
<td>0.449</td>
<td>0.0761</td>
<td>0.373</td>
<td>4.9</td>
<td>5.9</td>
</tr>
<tr>
<td>7.5(^b)</td>
<td>1.06</td>
<td>0.927</td>
<td>0.967</td>
<td>10.4</td>
<td>11.4</td>
</tr>
<tr>
<td>8.1(^b)</td>
<td>1.51</td>
<td>0.166</td>
<td>1.34</td>
<td>8.1</td>
<td>9.1</td>
</tr>
<tr>
<td>7.5(^c)</td>
<td>0.404</td>
<td>0.0927</td>
<td>0.311</td>
<td>3.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

\(^a\)CL = (INITIAL AMOUNT)/(AUC) per fixed experimental volume of 12.2 mL.

\(^{cl_{TOTAL}} = CL_{BUF} + CL_{LIP}\).

\(^b\)Exptl. condition 1 in Table 1.2

\(^c\)Exptl. condition 4 in Table 1.2
TABLE 1.4 Particle Sizes of Cetrimonium-containing Liposomes, Apparent First-order Rate Constants ($k$ in min$^{-1}$) for Loss of $1.08 \times 10^{-3}$ M p-Nitrophenyl Acetate and the Ratios of These Rate Constants to Those in the Corresponding Buffer Controls ($k_B$, 40°C, $\mu = 0.4$). Experimental Conditions$^a$: (1) Liposomal Suspensions, (2) Supernatant after Ultracentrifuging, and (3) Filtered Supernatant.

<table>
<thead>
<tr>
<th>Exptl. Condition</th>
<th>pH</th>
<th>Total Count</th>
<th>Mean Diameter (µM)</th>
<th>% in 1.5-4.0 µM Range</th>
<th>$10^3k$ (min$^{-1}$)</th>
<th>RATIO $(k/k_B)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.5</td>
<td>27,504</td>
<td>2.60</td>
<td>78</td>
<td>7.20</td>
<td>0.8</td>
</tr>
<tr>
<td>1</td>
<td>7.0</td>
<td>25,423</td>
<td>2.53</td>
<td>75</td>
<td>3.80</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>529$^c$</td>
<td>----</td>
<td>----</td>
<td>6.70</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>116$^c$</td>
<td>----</td>
<td>----</td>
<td>6.70</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$See text for details.

$^b$Count per 200 µL using an Elzone Model 80XY counter following a 4 x 10$^3$ fold sample dilution.

$^c$Average background count = 100 to 200.
TABLE 1.5: Experimental Conditions and Rate Constants for Loss of 1.08 x 10⁻³ M p-Nitrophenyl Acetate Stearylamine Suspensions in Phosphate Buffers, μ = 0.4, 40°.

<table>
<thead>
<tr>
<th>pH²</th>
<th>Before</th>
<th>After</th>
<th>TOTAL STEARYLAMINE CONCENTRATION 10⁻³ M⁻¹</th>
<th>RATE CONSTANT 10⁻³ kᵦ in min⁻¹</th>
<th>10⁻³ kₛᵤₚ in min⁻¹</th>
<th>TLC AMIDE TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.88</td>
<td>6.90</td>
<td>2.5ᵇ</td>
<td>6.00</td>
<td>5.70</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>6.89</td>
<td>6.90</td>
<td>4.8</td>
<td>6.00</td>
<td>6.30</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>7.66</td>
<td>7.64</td>
<td>2.4ᵇ</td>
<td>7.60</td>
<td>10.4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8.27</td>
<td>8.15</td>
<td>1.2</td>
<td>14.9</td>
<td>20.2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8.37</td>
<td>8.20</td>
<td>2.6ᵇ</td>
<td>17.0</td>
<td>23.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8.46</td>
<td>8.35</td>
<td>3.6</td>
<td>19.4</td>
<td>24.3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8.52</td>
<td>8.40</td>
<td>4.7</td>
<td>21.3</td>
<td>27.7</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

aMeasured before and after reaction.

bApproximately the concentration of stearylamine used in liposome study.

cCalculated using Eq. 1.4.

dAfter ≥ 90% of reaction is complete.
TABLE 1.6 Summary of Ratios of Total Clearance Values\textsuperscript{a} for the Loss of p-Nitrophenyl Acetate in Various Experimental Systems\textsuperscript{b} Relative to Values in the Corresponding Buffer Controls (40°C) and Test Results for N-Stearylacetamide.

<table>
<thead>
<tr>
<th>EXPERIMENTAL</th>
<th>pH</th>
<th>(10^3) COUNT per (\mu)L</th>
<th>CLEARANCE RELATIVE TO CONTROL</th>
<th>AMIDE TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEARYLAMINE LIPOSOMES</td>
<td>5.8 - 8.1</td>
<td>6.0(±0.6)</td>
<td>8.0 (2.8)\textsuperscript{c}</td>
<td>(+)</td>
</tr>
<tr>
<td>AFTER LIPOSOME REMOVAL</td>
<td>7.5</td>
<td>BACKGROUND</td>
<td>1.0</td>
<td>(-)</td>
</tr>
<tr>
<td>STEARYLAMINE-DMPC\textsuperscript{d} LIPOSOMES</td>
<td>7.5</td>
<td>4.5</td>
<td>4.4</td>
<td>(+)</td>
</tr>
<tr>
<td>STEARYLAMINE SUSPENSIONS</td>
<td>6.9</td>
<td>----</td>
<td>1.0</td>
<td>(-)</td>
</tr>
<tr>
<td>CETRIMONIUM LIPOSOMES</td>
<td>7.0 - 7.5</td>
<td>5.3\textsuperscript{c}</td>
<td>0.7\textsuperscript{c}</td>
<td>(-)</td>
</tr>
<tr>
<td>AFTER LIPOSOME</td>
<td>7.0</td>
<td>BACKGROUND</td>
<td>1.0</td>
<td>(-)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}CL = \((\text{INITIAL AMOUNT})/(\text{AUC})\) per 12.2 mL. For monoexponential loss the ratios of the rate constants equal the clearance ratios.

\textsuperscript{b}See text for details.

\textsuperscript{c}\(\bar{x}(SD)\)

\textsuperscript{d}Dimyristoyl phosphatidylcholine used in place of L-\(\alpha\)-phosphatiylcholine.
Figure 1.1: A typical size distribution profile of L-α-phosphatidylcholine liposomes obtained using the Elzone particle size analyser.
Figure 1.2: Ultraviolet absorption spectra for $1.04 \times 10^{-5}$ M p-nitrophenyl acetate and $8.78 \times 10^{-5}$ M p-nitrophenol in 0.5 M formic acid containing 80% methanol.
Figure 1.3: Ultraviolet absorbance at 271 (□) and 315 nm (○) versus p-nitrophenyl acetate concentration in 0.5 M formic acid containing 80% methanol.
Figure 1.4: Ultraviolet absorbance at 271 (□) and 315 nm (○) versus p-nitrophenol concentration in 0.5 M formic acid containing 80% methanol.
Figure 1.5: Catalytic effect of buffer on the observed rate constants for loss of p-nitrophenylacetate at 40°, =0.4 in citrate buffer A, pH 4.10; phosphate buffers B, pH 4.85; C, pH 6.92; D, pH 7.56; E, pH 8.13; Tris(hydroxymethyl)aminomethane buffers F, pH 7.7; G, pH 8.35; and Borate buffer H, pH 9.12.
Figure 1.6: Semilogarithmic plots for the fraction of p-nitrophenyl acetate remaining as a function of time at 40°C in the presence liposomes containing stearylamine. A: pH 5.83 (*) B: pH 7.0 (△) pH 7.53 (○); pH 8.09 (○).
Figure 1.7: pH rate profile of p-nitrophenyl acetate in the absence (curve A) and presence (curve B) of buffer; in buffered L-α-phosphatidylycholine: stearylamine liposomal suspension (curve C= initial phase, curve D=terminal phase).
Figure 1.8: Semilogarithmic plots for the fraction of p-nitrophenyl acetate remaining as a function of time in the presence of cetrimonium ion-containing liposomes ( ), and in control buffers (dashed line) at pH 7.5, 40°C.
Figure 1.9: The percent loss of p-nitrophenyl acetate (PNPA) and percent formation of p-nitrophenol (PNP), acetic acid (Ac) and stearylacetamide (Am) as a function of time at pH 7.53, 40°C. Circles (o): experimental data; Solid curve(---): drawn from data generated employing the fourth order Runge Kutta numerical method.
Figure 1.10: Titration of surface amino groups on liposomes containing stearylamine. A: pH of the stearylamine-containing liposomal suspensions versus the volume of 0.01 N HCl added; B: the first derivative (ΔpH/ΔV) versus the volume of 0.01 N HCl added.
Figure 1.11: Bronsted plot at 40°C relating buffer catalytic rate constants (k) to the dissociation constants of their conjugate acids (pKa); p represents the number of equivalent protons which can be transferred from the conjugate acid and q the number of sites on the general base which will accept a proton.
CHAPTER II
INHIBITION OF DRUG HYDROLYSIS BY LIPOSOMAL ENCAPSULATION OF STABLE DRUG SOLUTIONS
This investigation demonstrates that it is possible to encapsulate stable ancitabine solutions in liposomes thus inhibiting hydrolysis of this prodrug upon subsequent redispersion in an environment where ancitabine is unstable. The degree of success was gauged using stabilization ratios which compared prodrug stability under identical conditions in the absence of liposomes to that in these liposomal dispersions. The ability of liposomes to protect ancitabine effectively was dependent on the phase transition temperature (PTT) of the phospholipid used in liposome preparation. Stabilization ratios as high as 22 were observed when the PTT of the phospholipids were 16 to 26°C higher than the ambient temperature. For a given phospholipid, stabilization decreases finally disappearing as the ambient temperature is increased until it equals the PTT. Liposomes composed of phospholipids with PTT values below the ambient temperature were ineffective in stabilizing encapsulated ancitabine. Liposomal integrity decreases above the PTT as evidenced by rapid, nearly complete, release of encapsulated ancitabine. Loss of stabilization when the encapsulated solution was prepared at the pH of the external medium, indicates that the previously observed protection was due primarily to maintenance of a pH gradient rather than to interactions of the prodrug with the liposomal membrane. A direct relationship was observed between the degree of ancitabine stabilization and the liposomal prodrug payload. Inclusion of cholesterol in liposomes produced a reduction in stabilization ratio the largest being from 22 to ~3.8.
INTRODUCTION

Among the various nucleoside derivatives investigated for their potential antitumor activity, cytarabine (arabinosylcytosine, ara-c) finds wide clinical use especially in the treatment of acute myelogenous leukemia in adults.\(^1\)-\(^3\) It exerts its action, following entry into leukemic cells where it is converted to the 5'-triphosphate (5-CTP).\(^4\) Although cytarabine is an active antileukemic agent, its short duration due to rapid enzymatic deamination (cytidine deaminase),\(^5\) necessitates the use of constant intravenous infusions or complex dosage schedules in therapy.

Ancitabine (cycloctydine, cyclo-c), an anhydro analogue of cytarabine has shown potential as a prodrug in preliminary human clinical trials.\(^6\),\(^7\) It is known that 45-93\% conversion of the prodrug occurs within only 1 h in human plasma\(^8\) where it undergoes non-enzyme catalyzed chemical hydrolysis to form cytarabine.\(^9\) In vivo, competitive excretion of intact ancitabine is known to limit its conversion.

This study investigated a unique approach to the prevention of ancitabine hydrolysis by using liposomal encapsulation maintain the prodrug in a stable environment. An acidic ancitabine solution, which is known to be stable, is encapsulated in liposomes. The vesicles are
then dispersed in alkaline media under conditions where the prodrug is susceptible to hydrolysis (Fig. 2.1). The rate constants for loss of ancitabine in these dispersions were compared to control studies where liposomes were absent. Significant stabilization would be expected when hydroxyl ion entry into the liposomes and diffusion of prodrug out into the alkaline medium (Fig 2.1, inset) are effectively restricted. In order to examine the various factors influencing stabilization, the effect of varying liposome composition, temperature, pH gradients and prodrug loading were investigated.

Stabilization of ancitabine in liposomes could have important in vivo implications. Parenteral administration of stable ancitabine solutions in liposomes could lead to increased plasma circulation time for ancitabine which could indirectly increase intracellular cytarabine concentrations. Additionally, increased transfer of ancitabine into leukemic cells is expected through phagocytosis of the liposomes and/or fusion of the liposomes to the leukemic cell walls. Subsequent hydrolysis of the ancitabine to cytarabine inside the cell may then provide concentrations of the drug which exceed those achieved by cytarabine administration.
EXPERIMENTAL

Materials: --L-α-phosphatidylcholine (PC), type V-E, from frozen egg yolk, in chloroform:methanol (9:1) solution; cholesterol (CHOL), and ancitabine (cyclo-c) were obtained from Sigma Chemical Co.. Dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylglycerol (DPPG) and distearoyl phosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids. Cytarabine (ara-C) was a gift from The Upjohn Co..

Preparation of Liposomes by the Reverse Phase Evaporation Method:--Liposomes were prepared by the following methods based on reports by Szoka and Papahadjiopoulos. The concentrations given are for the preparation of 1 mL of liposomal suspension. The amount of phospholipid (PC, DMPC, DPPC, DSPC) used to prepare the vesicles was 66 μmoles. In some studies either 33 μmoles of cholesterol or 14 μmoles of phosphatidylglycerol was also incorporated. The lipid was placed in a 50 mL round bottom flask and the chloroform solvent (when PC was used) removed using a rotary evaporator. Diethyl ether was then added (3 mL) to dissolve the lipids. When either DPPC or DSPC was employed, ~1.5 mL of chloroform was introduced to ensure dissolution prior to addition of ether. The appropriate aqueous buffer (1 mL)
containing $4 \times 10^{-2}$ M (unless otherwise specified) ancitabine was added. At this point in the procedure the ratio was 1 part aqueous phase : 3 parts diethyl ether : ~1.5 parts of chloroform (when necessary). The head space above the lipid was intermittently flushed with nitrogen during the preparation. The mixture was sonicated in a cylindrical bath type sonicator (Model W-375; Heat Systems-Ultrasonics Inc.) for 3-5 min at temperatures close to the phase transition temperature (PTT) of the phospholipid. Following sonication a milky white homogeneous emulsion was obtained.

During the sonication process it was observed that some round bottom flasks facilitated the formation of stable emulsions better than others. The reason for this was not clear but it may be due to thinner wall structure in these flasks.

The lipid emulsion was then subjected to rotary evaporation at 30°C for PC and DMPC, at 41°C for DPPC and at 55°C for DSPC. Initially the organic phase was drawn off slowly with low vacuum. Removal of most of the organic phase results in a viscous preparation which foams vigorously. The flask was periodically vented and the vacuum progressively increased until foaming was minimal and a liquid was formed. The remaining volume was measured and the aqueous phase lost during the preparation was replaced.

A 50 µL sample was taken for analysis of total ancitabine concentration in the liposomal suspension. The remaining liposomal suspension was dialyzed against 100 times its volume of 0.15 M acetate buffer
(300 mOsm/L), at 4°C, for 24 h with two changes of external dialysis fluid at approximately equal intervals. In selected studies ultracentrifugation (Beckman L5-50B Ultracentrifuge) was employed after dialysis to remove any residual free ancitabine. A 1 mL aliquot of the liposomal suspension was diluted with 7 mL of isoosmolar buffer and ultracentrifuged at 41,000 rpm for 15 minutes at 4°C. The supernatant was removed (~7 mL) and analyzed for free ancitabine. The liposomal pellet was redispersed in an equal volume of fresh isoosmolar buffer and centrifugation repeated. Prior to each centrifugation, liposomal suspensions were analyzed for total ancitabine concentration. The ultracentrifugation was repeated three times which was found to be adequate to remove most of the free prodrug. The free prodrug in the final liposomal suspension was less than 5 percent of the total.

By comparing studies involving removal of free prodrug by dialysis to those which were followed by ultracentrifugation it was determined that dialysis alone removed more than 95 percent of the free prodrug.

In studies where rapid preparation of liposomes was necessary, unencapsulated ancitabine was removed using only ultracentrifugation. The overall time involved to obtain final liposomal preparations using this method was ~1 hour.

The percent ancitabine encapsulated was calculated using

\[ \% \text{ encapsulated} = 100 \frac{C_a}{C_b} \]  

(2.1)
where $C_a$ and $C_b$ are the concentrations of ancitabine after and before dialysis and/or centrifugation respectively and

$$\text{loading factor} = \frac{\text{moles prodrug encapsulated}}{\text{moles lipid}} \quad (2.2)$$

The size distribution for each of the liposomal suspensions was determined with an Elzone model XY particle size analyzer following a $1 \times 10^5$ fold dilution of the liposomal suspension with isoosmolar sodium chloride solution (Fig. 2.2 and 2.3; Table 2.1).

**Analytical Method:** --Spectrophotometric (Model Gilford 250 spectrophotometer) analyses of ancitabine and its hydrolysis product cytarabine in buffered aqueous solutions and following encapsulation in liposomes were carried out as follows.

Studies investigating the stability of liposome encapsulated ancitabine involved analysis for both prodrug and drug. A 0.3-0.5 mL aliquot of the reaction mixture was added to 5.0 mL of a 0.2 M HCl in methanol solution to quench hydrolysis of ancitabine and dissolve the liposomes. Samples were stored in the refrigerator and warmed to room temperature before determining their absorbance. Precipitation of the lipid occurred when samples containing DSPC were stored in the cold. Warming these samples with shaking resulted in a clear solution. Absorbance was measured against reference solutions prepared in a similar manner without liposomes and ancitabine. No loss of ancitabine or cytarabine was observed on cold storage for periods of 48 hours.
The absorbance of ancitabine and cytarabine solutions were measured at their UV maxima, of 260 and 285 nM respectively (Fig. 2.4). The total absorbances at the two wavelengths were used to derive

\[
10^5 C_1 = 10.8 A_{260} - 3.31 A_{285} \quad (2.3)
\]

\[
10^5 C_2 = 7.91 A_{285} - 2.44 A_{260} \quad (2.4)
\]

from which concentration of ancitabine \((C_1)\) and cytarabine \((C_2)\) in the final dilutions were calculated. The accuracy of the method was tested by determining the concentrations of ancitabine and cytarabine in known mixtures. Beer's law plots in 0.2 M HCl in methanol (Figs. 2.5, 2.6) gave molar extinction coefficients of \(10.2 \times 10^3\) at 260 nM and \(3.17 \times 10^3\) at 285 nM for ancitabine and \(4.28 \times 10^3\) and \(14.0 \times 10^3\) for cytarabine.

**Hydrolysis of Ancitabine Encapsulated in Liposomes and in Aqueous Buffers:**

**Buffers:** The hydrolytic susceptibility of ancitabine solutions encapsulated in liposomes was tested as follows. To 1 part of suspension of liposomes containing an encapsulated solution of ancitabine in acetate buffer (pH 4.7) was added 9 parts of isoosmolar bicarbonate buffer (pH 9.54). Aliquots (0.3-0.5mL) of this reaction mixture maintained at constant temperature were removed as a function of time and analyzed for total ancitabine and cytarabine. The head space above the reaction mixture was flushed with nitrogen after withdrawing each sample for analysis. In control studies the hydrolysis of ancitabine was examined in similar buffers in the absence of liposomes.
Constant temperatures were maintained using thermostatically controlled water baths (Model ST, Sargent Thermomonitor). In studies requiring temperatures below 25°C, water baths containing cooling coils were used (Model FK, Haake Instruments Inc.).

Temperature Dependent Release of Ancitabine from Liposomes:
--Prodrug release from liposomes was studied using the following procedures. To one part of the suspension of liposomes containing encapsulated ancitabine was added 9 parts of isoosmolar 0.15 M acetate buffer (300 mOsm/L) maintained at the desired temperature. Aliquots of the suspension were removed at the end of 5 and 15 minutes. Each aliquot was divided into two portions. One part (0.3-0.5 mL) was taken for estimation of total ancitabine. The other part (0.6 mL) was added to an equal volume of ice cold isoosmolar 0.15 M acetate (pH 4.7) buffer and subjected to high speed centrifugation (Sorval superspeed RC-2B centrifuge) at 20,000 rpm for 30 minutes. Following centrifugation a 0.6 mL aliquot of the supernatant was diluted 6 fold with 0.2 M HCl in methanol and analyzed for ancitabine. The percent released at the end of 5 and 15 minutes was calculated using

\[
\% \text{ released} = 100\% \ D \left( \frac{C_S}{C_t} \right) \quad (2.5)
\]

where \( C_S \) is the concentration of ancitabine in the supernatant, \( C_t \) is the total concentration in the liposomal suspension and \( D \) is the dilution factor.
RESULTS

The hydrolysis of acidic ancitabine solutions (pH ~4.7) encapsulated in liposomes was monitored following redispersion of the vesicles in an alkaline environment (pH ~9.5). The ability of liposomes to protect ancitabine was evaluated by comparing the rate constants for hydrolysis in controls under identical conditions in the absence of liposomes to those obtained in the liposomal suspensions.

Most systems were described by first order rate constants obtained from

\[ \ln F = \ln 1 - kt \]  

(2.6)

where \( t \) is the time, \( F \) is the fraction ancitabine at time \( t \), and \( k \) is the observed first order rate constant which is denoted as \( k_B \) when conducted in control buffers and \( k_L \) in liposomal suspensions (Tables 2.2-2.7).

Tables 2.2, 2.3 and 2.6 also list values for the rate constants \( \alpha \) and \( \beta \) for those cases where biphasic loss of the prodrug was observed. These values were obtained using

\[ F = f_A e^{-\alpha t} + f_B e^{-\beta t} \]  

(2.7)
where $F$ is the fraction remaining at time $t$, $f_A$ and $f_B$ are the ancitabine fractions lost in accordance with the rapid ($\alpha$) and the slow ($\beta$) rate constants respectively. The values for $f_A$, $f_B$, $\alpha$ and $\beta$ were obtained using nonlinear regression.

The ratio of the rate constant in the control ($k_B$) to that in the corresponding liposomal suspension ($k_L$ or $\beta$) is defined as the stabilization ratio where

$$\text{stabilization ratio} = \frac{k_B}{k_L} \text{ or } \frac{k_B}{\beta} \quad (2.8)$$

The sum of the ancitabine and cytarabine concentrations throughout all reactions was equal to the initial concentration of the prodrug.

**Cholesterol-Free Liposomes:** -- Table 2.2 shows encapsulation efficiencies, loading factors, hydrolysis rate constants and stabilization ratios for ancitabine encapsulated in liposomes prepared using various phospholipids. The phase transition temperature (PTT) for each of the phospholipids is also listed. Encapsulation efficiencies in these liposomes were found to be relatively constant at ~30% except in DSPC liposomes where encapsulation was only 9.91%. Loss of encapsulated ancitabine in the various liposomes is shown in Fig. 2.7. First-order hydrolysis of ancitabine was observed in DMPC, DPPC, and DSPC liposomes (Eq. 2.6) under the conditions in Table 2.2. Loss of the prodrug was biphasic in PC liposomes (Eq. 2.7) presumably due to the
high permeability of these vesicles. Rate constants for hydrolysis of ancitabine in liposomes and in control buffers were plotted against the PTT values of the phospholipid (Fig. 2.8). Bar graphs showing the corresponding stabilization ratios as a function of the PTT values are represented in Fig. 2.9. Significant stabilization ratios ($k_B/k_L = 22$) were achieved in liposomes containing phospholipids which have a PTT greater than the temperature of the study (25°C). However DMPC (PTT=23°C) and PC (PTT=4°C) do not show any increase in stability as indicated by their stabilization ratios of ~1.0.

**Liposomes Containing 33 Mol% Cholesterol:** --Encapsulation efficiency in DPPC:DPPG:CHOL liposomes containing 33 mol% cholesterol was higher than that obtained in vesicles composed of a single phospholipid and cholesterol (Table 2.3). This may be due to the negative charge on these liposomes which probably allows a higher capture of aqueous volume. All first-order hydrolysis rate constants for ancitabine in cholesterol containing liposomes (Table 2.3) were obtained using Eq. 2.6 except in the PC liposomes where loss of the prodrug was again biphasic (Eq. 2.7).

Semilogarithmic plots for the hydrolysis of encapsulated ancitabine as a function of time are illustrated in Fig. 2.10. The rate constants for hydrolysis of prodrug encapsulated in these liposomes and in control buffers are plotted against the PTT values of the phospholipids used in liposome preparation in Fig. 2.11. Figure 2.12 compares the
stabilization ratios in the presence of cholesterol to those in its absence. It is observed that the ratios in the presence of cholesterol increase less dramatically with an increase in the PTT values of the phospholipids than those in the absence of cholesterol. Maximum stabilization (~6 fold increase) was achieved with the DSPC:CHOL vesicles. The dramatic 22-fold increase in ancitabine stabilization observed with DPPC and DSPC liposomes was reduced to only a 4-to 6-fold increase in the presence of cholesterol.

**Effect of Temperature:** --The temperature dependencies of $k_B$, $k_L$ and the stabilization ratios in DPPC and DPPC:DPPG:CHOL liposomes are presented in Table 2.4 and Figs. 2.13-2.16. The rate constants for hydrolysis of ancitabine encapsulated in DPPC liposomes and those in control buffers converge at approximately the PTT when plotted as a function of temperature (Fig. 2.13). The decrease in the stabilization ratio with an increase in temperature is shown in Figure 2.14. The ratios approach unity at temperatures above the PTT of DPPC where the absence of stabilization was noted.

In contrast to the results in Figure 2.13, the rate constants for the hydrolysis of ancitabine encapsulated in DPPC:DPPG:CHOL liposomes and those in the control buffers do not converge when plotted as a function of temperature (Fig. 2.15). The stabilization ratios remained relatively constant at ~3.7 (Fig. 2.16) throughout the temperature range of the study.
Figure 2.17 summarizes the behavior of the stabilization ratios in the various liposomes when hydrolysis studies were conducted ~1-5°C above and below the PTT of the phospholipid used in the vesicles. The stabilization ratio shown for DPPC below its PTT is approximately 6 (5°C below its PTT of 41°C) whereas it was 22 at 25°C (16°C below the PTT).

At temperatures below the PTT increasing the temperature of the study to approach the PTT results in a corresponding decrease in the stabilization ratios. As discussed with respect to Fig. 2.14, the cholesterol-free liposomes exhibited no stabilizing effect above the PTT where the ratios are ~1 but significant increases were observed below the PTT in each case. In contrast, such marked differences in ratios above and below the PTT values are not apparent in the liposomes containing cholesterol(Fig. 2.17, D).

The percentage of ancitabine released in 5 minutes from DPPC and DPPC:CHOL liposomes as a function of temperature is presented in Figs. 2.18 and 2.19. Similar results were obtained for 15 minute release studies. The percentage released from DPPC liposomes (Fig. 2.18) showed a sharp increase at the PTT value (41°C). The maximum release obtained above the PTT varied from 70-80%. The addition of 33 mol% cholesterol to the phospholipids during liposome preparation resulted in vesicles which showed greater release of pro-drug at lower temperatures with a gradual increase over a broad temperature range reaching a maximum of 25% release in 5 minutes at 50°C (Fig. 2.19)
**Influence of the Amount of Cholesterol:** --Encapsulation efficiencies, loading factors, hydrolysis rate constants and stabilization ratios for ancitabine in DPPC liposomes containing varying amounts of cholesterol are described in Table 2.5. It is observed that the percentage encapsulation is relatively constant in the various liposomes independent of their cholesterol content. Addition of 16.6 mol% cholesterol to DPPC during liposome preparation results in vesicles which exhibit a sharp decrease in encapsulated prodrug stability relative to cholesterol-free liposomes. The stabilization ratio declines from 22.4 to ~3.8 as the cholesterol content increases from 0 to 16.6 mol% as shown in Fig. 2.20. Further increasing cholesterol content from 16.6-50 mol% does not produce a significant change in the stabilization ratio.

**Varying the Internal or External pH:** --The effect of altering either the internal or external pH of DPPC liposomes on the stability of encapsulated prodrug was investigated (Table 2.6). Suspensions of liposomes containing Tris-buffered prodrug solutions (pH = 8.62) were prepared employing previously described rapid procedures. Methods to initiate kinetic studies remained the same. Biphasic loss of ancitabine was observed under this condition and analyzed using Eq. 2.7. The rate constant \( \beta \) was ~3 fold slower than the control rate constant \( k_B \) while \( \alpha \) was 2.5 times faster. However, the contribution of the \( \alpha \) phase to the total AUC was less than 5 percent. The stabilization ratio was reduced from 22.4 (pH of internal medium = 4.7) to 3.05 (Fig. 2.21).
The stability of encapsulated prodrug was also examined under various external conditions while the internal medium was maintained constant (acetate buffer; pH = 4.7). Figure 2.22 shows that $k_L$ values decrease as the pH of the external medium is reduced from 9.4 to 7.3.

**Influence of Prodrug Loading:** Table 2.7 shows that the loading factor increases as a function of the initial concentration of the ancitabine solution used during formation of DPPC liposomes. In the range studied, the increase in loading factor was a linear function of this concentration with the slope of the linear regression line equal to 4.45 (Fig. 2.23).

Hydrolysis studies at pH 9.4, 25°C, indicate that the stabilization ratio is directly proportional to the loading factor for ancitabine (Fig 2.24). The equation for this linear regression line is

$$k_B / k_L = -3.19 + 136.97 \text{ (loading factor)}$$

(2.9)
DISCUSSION

Ancitabine is a water soluble prodrug (MW = 261.7) which does not appear to associate with liposomal membranes. It is most stable in acidic media and relatively unstable under alkaline conditions. These properties make it an ideal candidate to test the ability of liposomes to maintain pH gradients selected to retard prodrug hydrolysis by encapsulating in acid and re-suspending the liposomes in an unfavorable alkaline pH.

Role of the Phase Transition Temperature: --The alkaline dispersion of ancitabine encapsulated as an acidic solution in liposomes composed of pure phospholipids showed that DPPC and DSPC vesicles significantly increased prodrug stability relative to control buffer. The stabilization ratio, $k_B/k_L$, was approximately 22 under the conditions given in Fig. 2.9. In contrast, stabilization was not exhibited by PC and DMPC liposomes (Fig 2.9). These hydrolysis studies were maintained at 25°C which is below the PTT values for DPPC (41°C)\(^{12,13}\) and DSPC (55°C).\(^{12,13}\) At temperatures below the PTT values it is known that the interactions between the hydrophobic acyl chains of adjacent phospholipid molecules become significant resulting in tightly ordered packing of the phospholipid molecules in the lipid bilay-
Therefore, the movement of OH\textsuperscript{-} ions into the DPPC and DSPC liposomes and/or diffusion of prodrug out of these liposomes into the external alkaline environment would be restricted. This is consistent with the significant stabilization observed for these liposomes at 25°C. In the case of PC and DMPC liposomes, 25°C is higher than their PTT values, thus providing sufficient energy to reduce the interactions between the phospholipids. This increased bilayer mobility is known to decrease bilayer thickness and increase the hydration per phospholipid molecule.\textsuperscript{13-16} With this enhanced movement of the matrix, increased permeability and diffusion would destroy the ability of the liposomes to protect ancitabine from hydrolysis.

**Role of Cholesterol:** Figure 2.12 compares stabilization ratios with and without the addition of 33 mol\% cholesterol. Both the DPPC and DSPC liposomes show reduced stabilization compared to those prepared without cholesterol. Previous studies have shown that interfacial phospholipid-cholesterol interactions reduce the hydrophobic interactions between adjacent phospholipid molecules thus increasing the fluidity of the lipid bilayer.\textsuperscript{17,18} Although the exact nature of the phospholipid-cholesterol interaction is not understood, it has been found that the planar sterol structure, the hydrophobic side chain at the C\textsubscript{17} position and the 3 β-hydroxy group are essential for the interactive process. The disruptive effect of cholesterol on the lipid bilayer, which allows an increase in the phospholipid acyl chain movement, would lead to increased permeability. This is consistent with
greater release of ancitabine obtained from the DPPC:CHOL liposomes at lower temperatures relative to vesicles containing DPPC alone (Figs. 2.18, 2.19).

**Temperature Dependence:** --Above the PTT, acid encapsulated ancitabine in DPPC liposomes showed no increase in stability relative to control buffers and ratios were approximately 1 (Fig 2.17). Below the PTT, stabilization ratios decreased with a rise in temperature (Fig 2.14). Reports containing X-ray diffraction and NMR data have indicated that molecular motion of the acyl chains in the lipid bilayer gradually increases with increasing temperature until significant molecular movement occurs at the PTT. Thus, below the PTT increased mobility of the lipid bilayer would increase permeability. This is consistent with the observed temperature-dependent decline in the stabilization ratio.

At temperatures above the PTT the absence of stabilization is probably due to loss of liposomal integrity. This is evidenced by the fact that during a 5 minute period, 70-80% of the prodrug was released from the liposomes above the PTT. The hydroxyl ion being smaller in size than the prodrug would be expected to have even greater permeability. Additionally, it is known that the order of permeability of the bilayer to various molecules is: anions > cations. These observations are consistent with previous reports that the movement of various molecules through the bilayer was dramatically increased near the
PTT.\textsuperscript{20,21} Thus above the PTT, free movement of both OH\textsuperscript{-} ions and prodrug through the lipid bilayers probably occurs resulting in the absence of stabilization.

In contrast, the addition of 33 mol\% cholesterol during liposome preparation results in vesicles exhibiting stabilization ratios of \textasciitilde3.7 which remain constant throughout the temperature range of the study (Fig. 2.16). Significant differences between the extent of stabilization achieved above and below the PTT of the phospholipid were absent. Cholesterol has been described as having an important 'modulatory' influence on the lipid bilayer.\textsuperscript{14,17} Below the PTT of the pure phospholipid it has a liquefying effect on the bilayer by reducing interaction between adjacent molecules. The bilayer therefore remains in a so-called 'intermediate fluid' condition both below and above the PTT. Thus the permeability of the bilayer is nearly independent of temperature. This is consistent with the constant stabilization ratios observed (Fig. 2.16).

**Maintenance of pH Gradients:** Changing the pH of the internal medium of DPPC liposomes from 4.7 to 8.6 reduces the stabilization ratio from \textasciitilde22 to \textasciitilde3. This study simulates the behavior that would be expected if instant significant decay of the pH gradient occurred when the liposomal systems were challenged. Therefore, it can be concluded that the dramatic increase in stabilization must be due at least in part to the successful maintenance of the pH gradient rather than a direct interaction with the liposomal structure.
Mechanism -- The observed rate constants for hydrolysis of ancitabine in DPPC and DSPC liposomes ($= 6.7 \times 10^{-4}$ min$^{-1}$) were greater than the theoretical values ($4.5 \times 10^{-7}$ min$^{-1}$) calculated for hydrolysis in the internal buffer ($pH = 4.7$). The observed value is approximately equal to that which would be expected at a $pH$ of 8.0 which is less than that observed at $pH$ 9.5 ($1.45 \times 10^{-2}$ min$^{-1}$). This implies some leakage of liposomal content and/or inward passage of external environment.

Therefore, the hydrolysis of prodrug may be represented by the processes in Scheme 2.1.

\[
\begin{align*}
(1) \quad & \text{OH}^-_{\text{ext}} \xrightarrow{k_1} \text{OH}^-_{\text{int}} \\
(2) \quad & \text{PD}_{\text{int}} + \text{OH}^-_{\text{int}} \xrightarrow{k_2} \text{D}_{\text{int}} + \text{H}_2\text{O} \\
(3) \quad & \text{PD}_{\text{int}} \xrightarrow{k_3} \text{PD}_{\text{ext}} \\
(4) \quad & \text{PD}_{\text{ext}} \xrightarrow{k_4} \text{D}_{\text{ext}} + \text{H}_2\text{O}
\end{align*}
\]

Scheme 2.1

Entry of hydroxyl ions from the external medium ($\text{OH}^-_{\text{ext}}$) into the internal medium ($\text{OH}^-_{\text{int}}$) of the liposomes results in hydrolysis of encapsulated ancitabine ($\text{PD}_{\text{int}}$) to cytarabine ($\text{D}_{\text{int}}$). Additionally, diffusion of encapsulated prodrug (process 3) to the external buffer ($\text{PD}_{\text{ext}}$) would result in hydrolysis to form free drug ($\text{D}_{\text{ext}}$) in accordance with process 4. The values of $k_1$ and $k_3$ would depend on the liposomal composition (addition of cholesterol etc.) and the temperature which also alters bilayer permeability.
The contributions of the individual processes to the overall hydrolysis of ancitabine in these liposomes were not determined. However, it is necessary that the \( k_1 \) and \( k_3 \) values are much less than \( k_B \) values for significant stabilization to be achieved. Due to its smaller size the permeation of hydroxyl ions through the liposome wall (process 1) is expected to be greater than that of ancitabine release (process 3). This is consistent with the fact that a significant reduction in the stabilization ratio was observed for DPPC when the temperature was increased from 25\(^\circ\) to 37\(^\circ\)C while prodrug release rate exhibited little change over this temperature range (Fig. 2.18). Therefore the loss in stabilization is attributed primarily to increased hydroxyl ion penetration rather than increase in loss of encapsulated prodrug to the external environment.

Increase in ancitabine loading into DPPC liposomes results in an increase in the stabilization ratio at 25\(^\circ\)C. This represents a decrease in the observed rate constant for hydrolysis of ancitabine in liposomal suspensions with increasing ancitabine payloads since the rate constant in control buffer remains unchanged. Increasing the initial amount of ancitabine loaded in liposomes is not expected to influence entry of hydroxyl ion into liposomes (process 1, Scheme 2.1). Therefore a reduction in \( k_3 \) value (process 3) may represent one explanation for the increased stability. As \( k_3 \) decreases the observed rate constant would increasingly reflect \( k_B \mathrm{OH}^- \) (process 2). This value is expected to lie somewhere between the rate constant for hydrolysis in the control buffer (pH 9.4) and the encapsulated internal buffer (pH 4.7).
Therefore the observed rate constant, which reflects the sum of these processes, would decrease as $k_3$ is reduced and $k_2\text{OH}^-$ takes on a predominant role.

In spite of the expected constancy of process 1, the relationship between the PD$_\text{Int}$ and OH$_\text{Int}$ concentrations (process 2) would change as the payloads are increased. The concentrations in process 2 would favor a second order reaction wherein the hydroxyl ion pool is supplied by process 1. If this is possible then the entire process in Scheme 2.1 should behave as a pseudo first order system with loss of prodrug occurring by the competing processes in 2 and 4. The Runge Kutta algorithm was used to simulate these experimental results using Scheme 2.1 as the model together with the initial conditions of the study and a variety of values for the rate constants. By changing only the payloads, it was possible to mimic the apparent first order data including a decreased rate constant value with an increasing payload.

Although the increased stability with increasing payloads may be achieved by changing either process 2 or 3, it is less likely that process 3 is the dominant factor since the increased payloads would not be expected to significantly reduce the release rate. Attempts to measure PD$_\text{ext}$ were erratic due to the low concentrations available for assay further implying that the large increase in stability is not likely to be due to a reduction in PD$_\text{Int}$ release.
REFERENCES


Table 2.1 -- Particle Sizes of Liposomes Composed of Various Lipids Used for Encapsulation of Ancitabine Solutions.

<table>
<thead>
<tr>
<th>Liposome Component</th>
<th>Mean (µM)</th>
<th>Median (µM)</th>
<th>Mode (µM)</th>
<th>Percent in 2.5-6.0 µM Range</th>
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<td>77.8</td>
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<td>3.10</td>
<td>2.97</td>
<td>2.84</td>
<td>73.9</td>
</tr>
<tr>
<td>DSPC</td>
<td>3.16</td>
<td>3.03</td>
<td>2.73</td>
<td>73.7</td>
</tr>
<tr>
<td>DSPC:CHOL</td>
<td>3.37</td>
<td>3.23</td>
<td>3.03</td>
<td>76.7</td>
</tr>
</tbody>
</table>

Abbreviations: PC = L-α-phosphatidylcholine  
DMPC = dimyristoyl phosphatidylcholine;  
DPPC = dipalmitoyl phosphatidylcholine;  
DSPC = distearoyl phosphatidylcholine;  
DPPG = dipalmitoyl phosphatidylycerol;  
CHOL = cholesterol.
Table 2.2 -- The Phase Transition Temperatures (PTT), Percentage Encapsulation, Loading Factors (Moles Ancitabine/Mole Lipid), Ancitabine Hydrolysis Rate Constants Following Dispersion of Cholesterol-Free Liposomes Containing Acidic Solutions (0.15 M Acetate Buffer at pH 4.7) in Bicarbonate Buffer (0.1 M at pH 9.5) and Stabilization Ratios (k_B/k_L or k_B/β) at 25°C.

<table>
<thead>
<tr>
<th>Liposome Component</th>
<th>PTT (°C)</th>
<th>%Encapsulation</th>
<th>Loading Factors</th>
<th>10^3k_L/min</th>
<th>k_B/k_L</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>23</td>
<td>29.7</td>
<td>0.176</td>
<td>14.1</td>
<td>1.03</td>
</tr>
<tr>
<td>DPPC</td>
<td>41</td>
<td>30.6</td>
<td>0.196</td>
<td>0.647</td>
<td>22.4</td>
</tr>
<tr>
<td>DSPC</td>
<td>55</td>
<td>9.91</td>
<td>0.119</td>
<td>0.677</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^3α</td>
<td>10^3β</td>
</tr>
<tr>
<td>PC</td>
<td>~4</td>
<td>31.1</td>
<td>0.191</td>
<td>132</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Abbreviations: are defined in Table 2.1

bSee Eq. 2.1
cSee Eq. 2.2
Table 2.3 -- Percent Encapsulation, Loading Factor (moles Ancitabine encapsulated/mole lipid), Ancitabine Hydrolysis Rate Constants Following Dispersion of Liposomes Containing Acidic Solutions (0.15 M Acetate Buffer at pH 4.7) in Bicarbonate Buffer (0.1 M at pH 9.5) and Stabilization Ratios (k_B/k_L, k_B/β) at 25°C in Phospholipid Liposomes Containing 33 Mol% Cholesterol (CHOL).

<table>
<thead>
<tr>
<th>Liposome Component</th>
<th>%Encapsulation</th>
<th>Loading Factors</th>
<th>10^3 k_L</th>
<th>k_B/k_L</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC:CHOL</td>
<td>26.1</td>
<td>0.151</td>
<td>7.85</td>
<td>1.85</td>
</tr>
<tr>
<td>DPPC:CHOL</td>
<td>28.3</td>
<td>0.162</td>
<td>3.80</td>
<td>3.82</td>
</tr>
<tr>
<td>DPPC:DPPG.CHOL</td>
<td>60.0</td>
<td>0.331</td>
<td>5.10</td>
<td>2.84</td>
</tr>
<tr>
<td>PC:CHOL</td>
<td>26.9</td>
<td>0.154</td>
<td>55.0</td>
<td>8.70</td>
</tr>
</tbody>
</table>

Abbreviations: are defined in Table 2.1.

bSee Eq. 2.1

cSee Eq. 2.2
Table 2.4 -- Rate Constants and Stabilization Ratios ($k_B/k_L$) as a Function of Temperature for Hydrolysis of Ancitabine Following Dispersion of Liposomes Containing Acidic Solutions (0.15 M. Acetate Buffer at pH 4.7) in 0.1 M Bicarbonate Buffer at pH 9.5 ($k_L$) and in Control Buffers at pH 9.5.

<table>
<thead>
<tr>
<th>Liposome Component</th>
<th>Temperature (°C)</th>
<th>$10^3k_B$ (min$^{-1}$)</th>
<th>$10^3k_L$ (min$^{-1}$)</th>
<th>$k_B/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>25.0</td>
<td>14.5</td>
<td>0.647</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>27.0</td>
<td>1.75</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>37.0</td>
<td>74.7</td>
<td>12.6</td>
<td>6.33</td>
</tr>
<tr>
<td></td>
<td>44.0</td>
<td>224</td>
<td>137</td>
<td>1.64</td>
</tr>
<tr>
<td>DPPC:DPPG:CHOL</td>
<td>25.0</td>
<td>14.5</td>
<td>5.10</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>27.0</td>
<td>5.92</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td>37.0</td>
<td>79.7</td>
<td>17.2</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td>40.2</td>
<td>108</td>
<td>37.2</td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td>44.0</td>
<td>228</td>
<td>65.7</td>
<td>3.50</td>
</tr>
</tbody>
</table>

$^a$Abbreviations are defined in Table 2.1
Table 2.5-- Percent Encapsulation, Loading Factor (Moles Ancitabine/Mole Lipid), Rate Constants for Hydrolysis of Ancitabine Following Dispersion of Liposomes Containing Acidic Solutions (Acetate Buffer, 0.15 M; pH 4.70) in Bicarbonate Buffer (0.1 M, pH 9.54) ($k_r$), and Stabilization Ratios ($k_B/k_L$) at 25°C in DPPC\textsuperscript{a} Liposomes Containing Varying Amounts of Cholesterol (CHOL)

<table>
<thead>
<tr>
<th>Cholesterol content (mol%)</th>
<th>% Encapsulation</th>
<th>Loading factor</th>
<th>$10^3k_{L1}$ (min$^{-1}$)</th>
<th>$k_B/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>30.6</td>
<td>0.196</td>
<td>0.647</td>
<td>22.4</td>
</tr>
<tr>
<td>16.6</td>
<td>29.7</td>
<td>0.144</td>
<td>3.80</td>
<td>3.82</td>
</tr>
<tr>
<td>33.3</td>
<td>31.6</td>
<td>0.135</td>
<td>3.72</td>
<td>3.90</td>
</tr>
<tr>
<td>50.0</td>
<td>37.8</td>
<td>0.144</td>
<td>3.77</td>
<td>3.85</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Abbreviation: defined in Table 2.1
Table 2.6 -- Rate Constants for Hydrolysis of Ancitabine Encapsulated in DPPC\textsuperscript{a} Liposomes ($k_L\alpha,\beta$) and Stabilization Ratios ($k_B/k_L$, $k_B/\beta$) When Vesicle Internal Medium is Changed Keeping External Medium Constant and External Medium is Changed Keeping Internal Medium Constant at 25°C.

<table>
<thead>
<tr>
<th>INTERNAL BUFFER</th>
<th>EXTERNAL BUFFER</th>
<th>LIPOSOMAL SUSPENSION</th>
<th>$10^3k_L$ min</th>
<th>$k_B/k_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACETATE 4.7</td>
<td>BICARBONATE 9.5</td>
<td>9.37</td>
<td>0.647</td>
<td>22.4</td>
</tr>
<tr>
<td>ACETATE 4.7</td>
<td>TRIS 8.6</td>
<td>8.52</td>
<td>0.611</td>
<td>----</td>
</tr>
<tr>
<td>ACETATE 4.7</td>
<td>PHOSPHATE 7.7</td>
<td>7.34</td>
<td>0.375</td>
<td>----</td>
</tr>
<tr>
<td>ACETATE 4.7</td>
<td>BICARBONATE 9.5</td>
<td>9.37</td>
<td>0.647</td>
<td>22.4</td>
</tr>
<tr>
<td>TRIS 8.6</td>
<td>BICARBONATE 9.5</td>
<td>9.47</td>
<td>34.2</td>
<td>4.75</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Abbreviations given in Table 2.1
Table 2.7 -- Initial Concentration of Ancitabine in the Aqueous Phase during DPPCa Liposome Preparation, Loading Factors (Moles Ancitabine/Mole Lipid), Rate Constants for Hydrolysis of Ancitabine Following Dispersion of Liposomes Containing Acidic Solutions (Acetate Buffer, 0.15 M; pH 4.70) in Bicarbonate Buffer (0.1 M, pH 9.54) at 25°C ($k_L$), and Stabilization Ratios ($k_B/k_L$).

<table>
<thead>
<tr>
<th>$10^2$ Initial Ancitabine Concentration</th>
<th>Loading factor</th>
<th>$10^3 k_L$ (min$^{-1}$)</th>
<th>$k_B/k_L$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.99</td>
<td>0.085</td>
<td>1.35</td>
<td>10.7</td>
</tr>
<tr>
<td>3.82</td>
<td>0.196</td>
<td>0.647</td>
<td>22.4</td>
</tr>
<tr>
<td>5.90</td>
<td>0.263</td>
<td>0.466</td>
<td>31.1</td>
</tr>
<tr>
<td>7.86</td>
<td>0.336</td>
<td>0.344</td>
<td>42.2</td>
</tr>
<tr>
<td>11.5</td>
<td>0.525</td>
<td>0.207</td>
<td>70.1</td>
</tr>
</tbody>
</table>

*Abbreviation: defined in Table 2.1*
Figure 2.1: Schematic representation illustrating liposomal encapsulation of drug (D) dissolved in an acidic medium followed by dispersion of the liposomes in an alkaline medium where the drug is less stable. The curve represents the pH-rate profile for the drug. The inset shows the likely diffusion processes which would reduce the ability of the liposome to protect the drug from alkaline degradation.
Figure 2.2: Typical result of a size distribution study showing the distribution of DMPC:CHOL liposomes obtained using the Elzone particle size analyzer.
Figure 2.3: Typical result of size distribution study showing the distribution of DPPC liposomes obtained using the Elzone particle size analyzer.
Figure 2.4: Ultraviolet absorption spectra for 8.6x10^{-5} M ancitabine (A) and 6.1x10^{-5} M cytarabine (B) in 0.2 M HCl methanolic solutions.
Figure 2.5: Ultraviolet absorbance at 260 (•) and 285 (○) nm versus concentration of ancitabine in 0.2 M HCl methanolic solutions.
Figure 2.6: Ultraviolet absorbance at 260 (*) and 285 (o) nm versus concentration of cytarabine in 0.2 M HCl methanolic solutions.
Figure 2.7: Semilogarithmic plots for the fraction of ancitabine remaining as a function of time following acidic encapsulation in PC (*), DMPC (o), DPPC (□), and DSPC (Δ) liposomes with subsequent dispersion in pH 9.4 buffer at 25°C. The control solution is bicarbonate (0.1 M):acetate (0.15 M) at a 9:1 ratio with a pH = 9.4 (dashed line).
Figure 2.8: Rate constants for the hydrolysis of ancitabine as a function of the phase transition temperatures of the phospholipid following encapsulation as an acidic solution in PC, DMPC, DPPC, and DSPC liposomes with subsequent dispersion in pH 9.4 buffer at 25°C. The control solution (solid line) is bicarbonate (0.1 M):acetate (0.15 M.) at a 9:1 ratio with a pH = 9.4.
Figure 2.9: Stabilization ratios \((k_B/k_L, k_B/\beta)\) for ancitabine encapsulated in PC (A), DMPC (B), DPPC (C), and DSPC (D) liposomes as a function of the phase transition temperature of the phospholipid in each type of vesicle.
Figure 2.10: Semilogarithmic plots for the fraction of ancitabine remaining as a function of time following acidic encapsulation in PC:CHOL (o), DMPC:CHOL (*), DPPC:DPPG:CHOL (Δ), DPPC:CHOL (o), and DSPC:CHOL (x) liposomes followed by dispersion at pH = 9.4, 25°C. The control solution is bicarbonate (0.1 M.):acetate (0.15 M.) at a 9:1 ratio with a pH = 9.4 (dashed line).
Figure 2.11: Rate constants for the hydrolysis of ancitabine as a function of the phase transition temperatures of the phospholipid following acidic encapsulation in PC:CHOL (A), DMPC:CHOL (B), DPPC:CHOL (C), and DSPC:CHOL (D) liposomes with subsequent dispersion in pH 9.4 buffer at 25°C. The control solution (solid line) is bicarbonate (0.1 M):acetate (0.15 M.) at a 9:1 ratio with a pH = 9.4.
Figure 2.12: Stabilization ratios $\left( \frac{k_B}{k_L}, \frac{k_B}{\beta} \right)$ for ancitabine encapsulated in PC (A), DMPC (B), DPPC (C), and DSPC (D) liposomes with (shaded histogram) and without (open histogram) cholesterol as a function of the phase transition temperature of the phospholipid in each type of vesicle.
Figure 2.13: Rate constants for the hydrolysis of ancitabine as a function of temperature following acidic encapsulation in DPPC liposomes with subsequent dispersion in buffer at pH = 9.4 (•). The control solution (○) is bicarbonate (0.1 M):acetate (0.15 M) at a 9:1 ratio with a pH = 9.4.
Figure 2.14: Stabilization ratios ($k_B/k_L$) as a function temperature for ancitabine encapsulated in DPPC liposomes as an acidic solution with subsequent dispersion at pH = 9.4. The PTT for DPPC is 41°C.
Figure 2.15: Rate constants for the hydrolysis of ancitabine as a function of temperature following acidic encapsulation in DPPC:DPPG:CHOL liposomes with subsequent dispersion in pH 9.4 buffer(s). The control solution (*) is bicarbonate (0.1 M):acetate (0.15 M) at a 9:1 ratio with a pH = 9.4.
Figure 2.16: Stabilization ratios ($k_R/k_L$) for ancitabine encapsulated in DPPC:DPPG:CHOL liposomes as a function of temperature. The dashed line represents the average ratio of (3.7).
Figure 2.17: Stabilization ratios ($k_B/k_L$, $k_R/\beta$) for ancitabine encapsulated in PC (A), DMPC (B), DPPC (C), and DPPC:DPPG:CHOL (D) liposomes above (diagonal shading) and below (vertical shading) the phase transition temperature of the phospholipid in each type of vesicle.
Figure 2.18: The percentage of ancitabine released during 5 minutes from DPPC liposomes into 0.15 M. Isoosmolar acetate buffer as a function of temperature. The phase transition temperature of DPPC is 41°C.
Figure 2.19: The percentage of ancitabine released during 5 minutes from DPPC:CHOL liposomes into 0.15 M isoosmolar acetate buffer as a function of temperature. The phase transition temperature of DPPC is 41°C.
Figure 2.20: Stabilization ratios (kB/kL) for ancitabine encapsulated in DPPC:CHOL liposomes as a function of the molar content (mol%) of cholesterol.
Figure 2.21: Stabilization ratios ($k_B/k_L$) for ancitabine encapsulated in DPPC liposomes. The pH of the encapsulated ancitabine solutions are 4.7 (A) and 8.6 (B) while the external medium was maintained at pH = 9.5.
Figure 2.22: Rate constants for the hydrolysis of ancitabine as a function of pH following acidic encapsulation in DPPC liposomes with subsequent dispersion in buffers in the pH range 7.7 to 9.5 at 25°C.
Figure 2.23: The increase in the loading factor for ancitabine (moles encapsulated/mole of lipid) in DPPC liposomes as a function of the concentration of the ancitabine solution employed during vesicle preparation at pH 4.7.
Figure 2.24: Stabilization ratios ($k_B/k_L$) for ancitabine encapsulated in DPPC liposomes as a function of the loading factor for ancitabine (moles encapsulated/mole of lipid) at 25°C.
CHAPTER III

SYNTHESIS AND STABILITY OF A COVALENTLY LINKED LIPOSOME-PHENOL ESTER MODEL PRODRUG
SUMMARY

This study demonstrates the feasibility of covalently bonding a drug to a liposomal wall component to act as a drug delivery system in the form of a potential liposome prodrug. The model compound chosen, p-nitrophenol, was esterified with the stearic acid present in liposomes employing the coupling agents 1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide (EDCI) and dicyclohexylcarbodiimide (DCC). A wide range of experimental conditions were tested and it was found that reactions conducted with EDCI in distilled water or with DCC in distilled water or phosphate buffer yielded >80% p-nitrophenyl stearate relative to the initial p-nitrophenol concentration. When EDCI was used in the presence of phosphate buffer a reduction in the ester formation (~40% yield) was observed. The time for ten percent degradation (T90) of the liposome ester was evaluated at 37°C in the pH range 1-11. A comparison between the T90 value at pH 9.5 for the liposome ester and that of an aqueous solution of a similar long chain ester indicates a significant increase in the stability of the liposomal ester. Studies examining the susceptibility of the liposome ester to esterase show a ~3 to 17 fold rate enhancement relative to the buffered solutions. At pH 7.3, 37°C, the observed T90 of 10 hours provides a preliminary indication of sufficient stability for the liposome prodrug to circulate intact in vivo.
INTRODUCTION

Liposomes have attracted considerable international interest based on their potential for possible use as vehicles for drug delivery\(^1-3\) and they continue to undergo intensive studies for this purpose.\(^4\) Previous reports have shown liposomes to be particularly useful to protect drugs from degradation in vivo,\(^5,6\) to increase drug circulation time in the blood,\(^7\) and to deliver drugs to specific sites in the body.\(^8-11\)

However, liposome drug delivery systems suffer from some serious limitations in vivo. They tend to concentrate in the reticuloendothelial system thus impeding delivery to other sites.\(^12\) They also show increased permeability and release their encapsulated contents on contact with plasma or serum.\(^12,13\) This has been attributed to interactions of the phosphatidylcholine with high density lipoproteins.\(^14,15\) Increasing the amount of cholesterol in the lipid bilayer has been only partially successful in overcoming the problem.\(^16\)

The present study investigates the feasibility of covalently bonding drugs with reactive moieties to functional groups on the surface of liposomes using coupling agents. It is expected that drugs anchored in this manner could remain with the liposomes during plasma circulation. The bonding of p-nitrophenol (chosen as a model compound) to
stearic acid present in liposomes in the presence of coupling agents 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDCI) and dicyclo-hexylcarbodiimide (DCC) was investigated under various conditions. A liposomal prodrug should contain an adequate payload and like any prodrug have sufficient stability to withstand premature conversion. Therefore, the stability of the liposome-nitrophenol ester was evaluated in buffer solutions at various pH values to estimate the pH for optimum storage stability and in the presence of esterase to determine its stability in the presence of enzyme-catalyzed hydrolysis.
EXPERIMENTAL

**Synthesis of Liposome-Nitrophenol Ester:** Liposomes were prepared from L-α-phosphatidylcholine (13-14 × 10^{-3} M) and stearic acid (6.5-7.0 × 10^{-3} M) in the molar ratio of 2:1 using the surface film-sonication method described previously. Nitrogen-saturated aqueous solvents and flushing of the headspace with nitrogen gas were employed throughout the entire procedure. The model compound selected for attachment to the liposomes was p-nitrophenol. This was covalently linked to the stearic acid present in the liposomes to form the ester p-nitrophenyl stearate (scheme 3.1) using the coupling agents dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDCI). Yields of p-nitrophenyl stearate were determined under various conditions of temperature, solvent and coupling agents to select suitable conditions for the reaction (Table 3.1).

Qualitative tests for formation of the ester were performed with 2.5x10 cm TLC plates (Silica Gel Uniplates, 250 uM; Analtech Inc.) which were developed with chloroform acidified with glacial acetic acid and visualised using iodine vapors. The $R_f$ value of the ester was 0.78. Sample preparation for the TLC test involved extraction
**Scheme 3.1**

\[ R-N=\overset{\text{N}}{=}C=\overset{\text{N}}{=}R' + \overset{\text{O}}{\text{HO-C-}}(\overset{\text{CH}_2}{\text{CH}_2})_{16}-\overset{\text{O}}{\text{CH}_3} \rightleftharpoons 1 \overset{\text{O}}{\text{R-N=\overset{\text{N}}{=}C-\overset{\text{NH}}{=}}R'} \]

**CARBODIIMIDE**  **STEARIC ACID**

EDCI, \( R = \text{C}_2\text{H}_5, R' = (\text{CH}_2)_3\text{NMe}_2 \)

DCC, \( R = R' = \text{C}_6\text{H}_{11} \)

\[ \overset{\text{O}}{\text{R-N=\overset{\text{C-}}{\overset{\text{N}}{=}C-\overset{\text{NH}}{=}R'}} + \overset{\text{OH}}{\text{p-NITROPHENOL}} \rightleftharpoons 2 \overset{\text{O}}{\text{p-NITROPHENYL STEARATE}} \]

\[ \overset{\text{O}}{\text{R-NH-C-}}\overset{\text{N}}{\overset{\text{NH}}{=}}\overset{\text{R'}}{\text{R'}} + \overset{\text{O}}{\text{p-NITROPHENYL STEARATE}} \]
of an aliquot of the liposomal suspension with chloroform, followed by separation and drying of the organic phase with sodium sulfate.

The amount of p-nitrophenol covalently bonded to stearic acid in liposomes was estimated by analyzing for the total p-nitrophenyl stearate in the suspension using UV spectroscopy. Prior to analysis, a 0.05-0.25 mL aliquot of the liposomal suspension was dissolved in 5.0 mL of 0.5 M formic acid in methanol and the UV spectrum recorded. Formation of the ester was indicated by the loss of the original peak at 313 nM and the appearance of a new peak at 269 nM. The total concentration of the ester and the unreacted phenol was obtained using the following analytical techniques.

The preparation of the liposome-nitrophenol product was carried out in distilled water at room temperature using EDCI as the coupling agent. The conditions for the reaction were based on preliminary screening studies (Table 3.1). Liposomes were prepared in 9 mL of nitrogen-saturated distilled water using L-α-phosphatidylcholine and stearic acid as described above. To initiate the reaction, a 0.2 mL aliquot of a 9×10⁻³ p-nitrophenol stock solution in methanol was added to the liposomal suspension followed by 20 mg of EDCI (11.6×10⁻³ M) and the reaction mixture was agitated overnight. The liposomal suspension was then ultracentrifuged (Beckman L5-50B Ultracentrifuge) at 41,000×g (=105,000 rpm), 4°C for 30 minutes to form a pellet. The supernatant (=7 mL) was removed and replaced with an equal volume of fresh distilled water and the procedure repeated. Each supernatant
was analyzed for ester and phenol. The liposomal pellet was then dispersed in the remaining aqueous solvent. This suspension was subjected to extensive dialysis for 20-24 hours against 200 mL of distilled water with 3 changes of external dialysis fluid at equal intervals. The dialysis bag was replaced with a new one after approximately 12 hours. Selected liposome p-nitrophenol ester suspensions were assayed for ester concentration (Table 3.3) after each stage of the preparation: (1) overnight agitation, (2) ultracentrifugation and (3) dialysis. Ten batches of liposome p-nitrophenol ester prepared according to the above procedures were combined and used for further studies.

**Analytical Method:** The assay for total p-nitrophenyl stearate and total p-nitrophenol in liposomal suspensions were performed spectrophotometrically (Beckman DU-7 spectrophotometer) as follows. Molar absorptivities for p-nitrophenyl stearate and p-nitrophenol in methanol containing 0.5 M formic acid at their UV maxima 269 and 313 nm (Fig 3.1) were $10.1 \times 10^3$ and $1.36 \times 10^3$ for the ester and $3.00 \times 10^3$ and $10.7 \times 10^3$ for the phenol (Figs. 3.2-3.3). Concentrations of the ester ($C_1$) and phenol ($C_2$) in the final dilutions were calculated using

$$10^5 C_1 = 10.3 A_{269} - 2.89 A_{313}$$  \hspace{1cm} (3.1)

$$10^5 C_2 = 9.98 A_{313} - 1.31 A_{269}$$  \hspace{1cm} (3.2)

derived from simultaneous equations for total absorbance, $A$, at 269 and 313 nM.
For stability studies of the liposomal-nitrophenol ester in buffers, a 0.3 mL aliquot of the liposomal suspension was added to 4.0 mL of 0.5 M formic acid in methanol (to dissolve the liposomes and quench the hydrolysis of the ester) and the absorbance was measured against a blank prepared in a similar manner. The susceptibility of the liposome ester to esterase catalysed hydrolysis was examined using identical analytical procedures except that aliquots of the liposomal suspension in the formic acid quench were centrifuged at 3200 rpm for 10 min followed by analysis of the supernatant.

**Stability in Buffers:** --The time for 10% degradation of the liposomal ester (T90) was estimated in various buffers in the pH range 1-11 at 37°C (Table 3.4). To initiate the reaction, 0.4 mL of the liposome ester suspension was dispersed in 3.6 mL of buffer previously equilibrated to 37°C and the mixture was agitated in a temperature controlled shaker bath (Forma Scientific model 2564). Aliquots of the reaction mixture were removed as a function of time and analyzed for total liposome ester and p-nitrophenol.

**Susceptibility to Esterase:** --Enzyme catalyzed hydrolysis of the liposome ester was studied in the pH range 7.5-9.5 at 37°C (Table 3.5). Pig liver esterase (carboxylic ester hydrolase, EC 3.1.1.1., Sigma) was standardized using p-nitrophenyl acetate as the substrate before being used. The enzyme preparation was diluted 100 fold and 0.1 mL
of this dilution added to a 1.6 mM solution of p-nitrophenyl acetate in phosphate buffer at pH 7.23, 25°C. Analysis of the p-nitrophenol produced was performed according to the UV method reported previously.\textsuperscript{17,18} Initial rate studies using the slopes of the concentration-time profiles were used to determine the relative enzyme activity which was defined in units/mL of enzyme preparation. One enzyme unit was defined as that amount which was found to hydrolyze 10 mL of a 1.6 mM p-nitrophenyl acetate solution at the rate of 1 umole/min at 25°C, pH 7.23.

To initiate the enzyme catalyzed reaction, 0.4 mL of ester suspension was diluted with 3.6 mL of buffer to which was added 25 units of the enzyme (~0.11 mL) using a microburette. A 0.3 mL aliquot of the reaction mixture was removed as a function of time and analyzed for total ester and phenol.
RESULTS AND DISCUSSION

Liposomal Ester Preparation: The reaction between stearic acid in liposomes and p-nitrophenol was initially examined under the various conditions described in Table 3.1. The formation of liposomal p-nitrophenyl stearate in the presence of carbodiimide coupling agents (EDCI or DCC) is illustrated in Scheme 3.1.\(^1\) The initial reaction between the carbodiimide and stearic acid results in a reactive intermediate (step 1) which is attacked by p-nitrophenol to give the ester and the urea derivative. The sum of the total ester and the excess p-nitrophenol concentration was equal to the initial concentration of p-nitrophenol. The percent p-nitrophenol reacted is defined by

\[
\text{% p-nitrophenol reacted} = 100 \times \frac{C_1}{C_1+C_2}
\]  

(3.3)

where \(C_1\) is the measured concentrations of ester and \(C_2\) the free phenol. The percent reacted (Eq. 3.3) is shown as a function of time (Figs. 3.7-3.12) for each of the conditions tested in Table 3.2. Reactions conducted in distilled water using EDCI at room temperature resulted in final yields of \(~80\%\) for the percentage p-nitrophenol reacted (Fig. 3.7). The identical reaction conducted under refrigerated temperatures, previously reported to give significant yields of cova-
lently bonded product\textsuperscript{20}, gave maximum values of \(\sim 60\%\) (Fig. 3.7) within the same time period. No significant difference in the maximum percentages (\(\sim 80\%\)) were obtained between DCC (condition 3, Table 3.1, Fig. 3.8) and EDCI reactions (condition 1, Table 3.1) conducted at room temperature.

Only 30\% of the p-nitrophenol was found to react in phosphate buffer at room temperature using EDCI (Fig. 3.9). This represents a 2-3 fold decrease in yield compared to the identical reaction in distilled water. When reactions in phosphate buffer were conducted under refrigerated conditions a similar reduction (Fig. 3.11) relative to distilled water (Fig. 3.7) was observed. The buffered medium, which has a pH = 7.7, has a higher hydroxyl ion concentration compared to the distilled water at pH \(\sim 5.1\). Hydroxyl ions might reduce yields by competing with stearic acid in step 1 or p-nitrophenol in step 2 of Scheme 3.1 or by increasing the hydroxyl-ion catalyzed hydrolysis of the ester after it is formed.

The maximum percentage of p-nitrophenol reacted did not change when phosphate buffer was used in the reactions involving DCC wherein values remained at \(\sim 80\%\) (Fig 3.11). This was the only significant contrasting behavior noted between the reactions involving EDCI and DCC. The higher lipid solubility of DCC may provide an increase in its effective reactive concentration within the liposomes. This would reduce the ability of hydroxyl ions to compete effectively with the carbodiimide in the lipid phase possibly explaining the higher effi-
ciency of this coupling agent in the buffered solution. Figure 3.12 summarises the percent p-nitrophenol reacted at the end of 3 h under the various conditions investigated.

Although the reactions using either EDCI or DCC in distilled water at room temperature were successful, EDCI was selected because the excess unreacted EDCI and the urea byproduct could be easily separated from the liposomes using dialysis and/or centrifugation owing to their high aqueous solubility.

**Liposomal Ester Stability:** --Stability studies involving hydrolysis of the liposomal ester were performed in various buffers in the pH range 1-11 at 37°C (Table 3.4). The concentration-time plots for ester were fit using the regression equation from SAS

\[ C_1 = W + X_t + Y_t^2 + Z_t^3 \]  

(3.4)

where \(C_1\) is the concentration of ester, \(W, X, Y, Z\) are the flexible parameters, and \(t\) is the time. The time for 10% hydrolysis of the liposome-ester (\(T_{90}\)) was calculated from the final equation for the curve of best fit using computer reiteration to solve for \(C_1 = 0.9 C_0\) for ester. A similar treatment based on phenol production gave the same results.

Figure 3.13 is semi-logarithmic plot of \(T_{90}\) values as a function of pH. A direct comparison between the \(T_{90}\) values for p-nitrophenyl stearate in liposomes to those in a simple aqueous solution was not
feasible owing to the low aqueous solubility of the ester which pre-
cluded the determination of its $T_{90}$ values. However, its stability may
be assumed to be similar to that of the more soluble long chain ester,
$p$-nitrophenyl octanoate. The $T_{90}$ value for the hydrolysis of
$p$-nitrophenyl octanoate in solution at $37^\circ$C, pH 9.5, calculated from a
previous report$^{22}$ is 1.5 min. If this value is approximately the same
for $p$-nitrophenyl stearate, then ester associated with liposomes ($T_{90} =
9.87$ h) effects a ~400 fold increase in stability relative to that in an
aqueous solution.

The $T_{90}$ values obtained for the liposomal ester at pH 8.12 and
8.45 in Tris buffers appear to be lower than the dashed curve show-
ing the trend of the other points in Figure 3.13. The enhanced loss
of the ester, $p$-nitrophenyl acetate, in the presence of liposomes dis-
persed in Tris buffers has been reported previously.$^{23}$ It is possible
that the buffer component tris(hydroxy methyl) amino methane could
act as a nucleophile$^{24}$ resulting in aminolysis of the liposome-ester
thus accounting for its lower stability.

Since several penicillin ester prodrugs are known to convert
instantly in the presence of esterase,$^{25-27}$ the susceptibility of the
liposome-ester to esterase-catalyzed hydrolysis was investigated in the
pH range 7.3-9.5 at $37^\circ$C (Table 3.4, Fig. 3.13). The purpose of
this study was not to predict drug release patterns in vivo but to
investigate the stability of the liposome-ester (seen as a model for
liposome prodrugs) in the presence of enzymes normally found in the
blood.
The formation of p-nitrophenol as a function of time increased as the pH increased (Fig. 3.14). Maximum hydrolysis was obtained at pH 9.5 where the $T_{90}$ values was 16 fold greater in the absence of esterase (Fig. 3.13). The $T_{90}$ value at pH 7.30, which is close to the pH for blood, is 10.5 h. The total in vivo circulation time for liposomes injected into the blood is reported to be approximately 8 h. The $T_{90}$ value (10.5 h) obtained for this model liposome ester compound provides a preliminary indication of sufficient resistance to hydrolysis to merit synthesis of a liposomal prodrug having a similar covalent linkage. If the ester circulates primarily intact, then the liposome prodrug in the blood would be available for drug release subsequent to phagocytic or adsorptive cellular uptake of the liposomes per se.
REFERENCES


Table 3.1--Experimental Conditions Employed to Esterify p-Nitrophenol With the Stearic Acid (≈ 15 μmoles) in Liposomes (Lecithin ≈ 30 μmoles).

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Temperature</th>
<th>Coupling Agent</th>
<th>Reaction Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ambient</td>
<td>EDCI</td>
<td>Distilled</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>2</td>
<td>Refrigerator (~5°C)</td>
<td>EDCI</td>
<td>Distilled</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>3</td>
<td>Ambient</td>
<td>DCC</td>
<td>Distilled</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>4</td>
<td>Ambient</td>
<td>EDCI</td>
<td>Phosphate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buffer, pH=7.7</td>
</tr>
<tr>
<td>5</td>
<td>Refrigerator (~5°C)</td>
<td>EDCI</td>
<td>Phosphate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buffer, pH=7.7</td>
</tr>
<tr>
<td>6</td>
<td>Ambient</td>
<td>DCC</td>
<td>Phosphate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buffer, pH=7.7</td>
</tr>
</tbody>
</table>

Abbreviations: EDCI = 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide; DCC = N,N' Dicyclohexylcarbodiimide.
Table 3.2 -- Percent p-Nitrophenol Reacted<sup>a</sup> with Stearic Acid in Liposomes Under Six Experimental Conditions<sup>b</sup>

<table>
<thead>
<tr>
<th>HOURS</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>10.1</td>
<td>9.51</td>
<td>10.9</td>
<td>5.05</td>
<td>5.5</td>
<td>2.96</td>
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<tr>
<td>0.5</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>25.5</td>
<td>18.9</td>
<td>9.42</td>
</tr>
<tr>
<td>1.5</td>
<td>67.2</td>
<td>35.1</td>
<td>69.5</td>
<td>32.1</td>
<td>23.8</td>
<td>23.4</td>
</tr>
<tr>
<td>2.0</td>
<td>73.7</td>
<td>41.5</td>
<td>76.4</td>
<td>34.0</td>
<td>27.3</td>
<td>42.0</td>
</tr>
<tr>
<td>3.0</td>
<td>79.0</td>
<td>56.4</td>
<td>80.9</td>
<td>32.5</td>
<td>33.8</td>
<td>72.0</td>
</tr>
<tr>
<td>4.0</td>
<td>80.4</td>
<td>58.7</td>
<td>83.1</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>5.0</td>
<td>81.4</td>
<td>61.0</td>
<td>81.6</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>6.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>28.1</td>
<td>39.4</td>
<td>82.5</td>
</tr>
<tr>
<td>8.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>27.0</td>
<td>42.4</td>
<td>83.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Equation 3.3

<sup>b</sup>Described in Table 3.1
Table 3.3 -- Amounts (µmoles) and Percent Yields\(^a\) of Liposome-Nitrophenol Ester at Each Stage of Preparation in the Reaction Between p-Nitrophenol and the Stearic Acid in Liposomes Using EDC\(^1\) as the Coupling Agent\(^b\) in Distilled Water at Room Temperature.

<table>
<thead>
<tr>
<th>TRIAL</th>
<th>overnight agitation µmoles</th>
<th>%yield</th>
<th>centrifugation µmoles</th>
<th>%yield</th>
<th>dialysis µmoles</th>
<th>%yield</th>
<th>% TNP remaining after dialysis(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.18</td>
<td>45.30</td>
<td>18.93</td>
<td>31.55</td>
<td>17.86</td>
<td>29.77</td>
<td>1.77</td>
</tr>
<tr>
<td>2</td>
<td>27.61</td>
<td>46.02</td>
<td>20.04</td>
<td>33.40</td>
<td>19.69</td>
<td>32.82</td>
<td>3.58</td>
</tr>
<tr>
<td>3</td>
<td>33.26</td>
<td>55.44</td>
<td>28.08</td>
<td>46.80</td>
<td>27.18</td>
<td>45.30</td>
<td>5.93</td>
</tr>
<tr>
<td>4</td>
<td>31.26</td>
<td>52.10</td>
<td>27.16</td>
<td>45.27</td>
<td>25.63</td>
<td>42.72</td>
<td>6.60</td>
</tr>
<tr>
<td>5</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>19.28</td>
<td>32.14</td>
<td>1.38</td>
</tr>
<tr>
<td>6</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>25.70</td>
<td>42.84</td>
<td>0.0</td>
</tr>
<tr>
<td>7</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>22.72</td>
<td>37.87</td>
<td>11.43</td>
</tr>
<tr>
<td>8</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>21.42</td>
<td>35.70</td>
<td>11.68</td>
</tr>
<tr>
<td>9</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>19.31</td>
<td>32.18</td>
<td>4.43</td>
</tr>
<tr>
<td>10</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>20.45</td>
<td>34.08</td>
<td>5.98</td>
</tr>
<tr>
<td>Mean</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>36.5±5.4</td>
<td>5.28±3.9</td>
<td>5.28±3.9</td>
</tr>
</tbody>
</table>

\(^a\) % yield = 100% X Holes PNFS formed/Holes stearic acid
\(^b\) Initial amounts (µmoles): Stearic acid = 120, p-Nitrophenol = 80 EDC1 = 100
\(^c\) % PNFS = ((Amount p-Nitrophenol/Amount Ester)×Amount p-Nitrophenol) X 100 following dialysis.
Table 3.4 -- Time for Ten Percent Hydrolysis (T90) of 1.1 x 10^{-3}M \( \text{p-Nitrophenyl Stearate} \) Present in Liposomes in Buffered Aqueous Solutions Without and With (E) Esterase at 37°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>CONCENTRATIONS x 10^2</th>
<th>T90 (HOURS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCl</td>
<td>NaCl</td>
</tr>
<tr>
<td>1.08</td>
<td>10.0</td>
<td>34.0</td>
</tr>
<tr>
<td>1.32</td>
<td>5.00</td>
<td>39.0</td>
</tr>
<tr>
<td>1.99</td>
<td>1.00</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_3\text{COOH} )</td>
<td>( \text{CH}_3\text{COONa} )</td>
</tr>
<tr>
<td>4.64</td>
<td>20.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>( \text{NaH}_2\text{PO}_4 )</td>
<td>( \text{Na}_2\text{HPO}_4 )</td>
</tr>
<tr>
<td>7.30</td>
<td>4.00</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>( \text{Tris H}^+ )</td>
<td>( \text{Tris} )</td>
</tr>
<tr>
<td>8.12</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>8.45</td>
<td>10.0</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>( \text{NaHCO}_3 )</td>
<td>( \text{Na}_2\text{CO}_3 )</td>
</tr>
<tr>
<td>9.47</td>
<td>12.0</td>
<td>3.00</td>
</tr>
<tr>
<td>9.55</td>
<td>18.0</td>
<td>4.50</td>
</tr>
<tr>
<td>9.61</td>
<td>24.0</td>
<td>6.00</td>
</tr>
<tr>
<td>9.97</td>
<td>5.00</td>
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</tr>
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<td>10.1</td>
<td>7.50</td>
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<td>10.7</td>
<td>0.750</td>
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</tr>
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<td>10.8</td>
<td>1.13</td>
<td>9.00</td>
</tr>
<tr>
<td>10.9</td>
<td>1.50</td>
<td>12.0</td>
</tr>
</tbody>
</table>
Figure 3.1: Ultraviolet spectra for $9.11 \times 10^{-9}$ M p-nitrophenyl stearate (A) and $7.97 \times 10^{-5}$ M p-nitrophenol (B) in 0.5 M formic acid methanolic solutions.
Figure 3.2: Ultraviolet absorbance at 269 (□) and 313 (○) nM versus the concentration of p-nitrophenyl stearate in 0.5 M formic acid methanolic solutions.
Figure 3.3: Ultraviolet absorbance at 269 (□) and 313 (○) nM versus the concentration of p-nitrophenol in 0.5 M formic acid methanolic solutions.
Figure 3.4: Ultraviolet absorption spectra in 0.5 M formic acid methanolic solutions showing disappearance of the p-nitrophenol maximum at 313 nM and formation of the ester maximum at 269 nM as a function of time. The spectra were obtained during the reaction between p-nitrophenol and the stearic acid in liposomes carried out in distilled water, room temperature, using EDCI as the coupling agent.
Figure 3.5: Ultraviolet absorption spectra in 0.5 M formic acid methanolic solutions showing disappearance of peak at 313 nM p-nitrophenol and formation of a new peak at 269 nM as a function of time. The spectra were obtained during the reaction between stearic acid in liposomes and p-nitrophenol in distilled water at refrigeration temperatures using (~5°C) EDCI as the coupling agent.
Figure 3.6: Ultraviolet absorption spectra in 0.5 M formic acid methanolic solutions showing disappearance of peak at 313 nM p-nitrophenol and formation of a new peak at 269 nM as a function of time. The spectra were obtained during the reaction between stearic acid in liposomes and p-nitrophenol in distilled water at room temperature using DCC as the coupling agent.
Figure 3.7: Percent p-nitrophenol reacted as a function of time during the reaction between p-nitrophenol and the stearic acid in liposomes carried out in distilled water at room temperature (□) and refrigeration temperature (○, ~5°C.) using EDCI as the coupling agent.
Figure 3.8: Percent p-nitrophenol reacted as a function of time in the reaction between p-nitrophenol and the stearic acid in liposomes using DCC as the coupling agent in distilled water at room temperature.
Figure 3.9: Percent p-nitrophenol reacted as a function of time in the reaction between p-nitrophenol and the stearic acid in liposomes using EDCI as the coupling agent in phosphate buffer at room temperature.
Figure 3.10: Percent p-nitrophenol reacted as a function of time in the reaction between p-nitrophenol and the stearic acid in liposomes using EDCI as the coupling agent in phosphate buffer at refrigeration temperature.
Figure 3.11: Percent p-nitrophenol reacted as a function of time in the reaction between p-nitrophenol and the stearic acid in liposomes using DCC as the coupling agent in phosphate buffer at room temperature.
Figure 3.12: Percent p-nitrophenol reacted after 3 h for the reaction between p-nitrophenol and the stearic acid in liposomes under the six conditions (1-6) described in Table 3.1.
Figure 3.13: Semilogarithmic plot of the time for ten percent hydrolysis ($T_{90}$ in hours) of $1.1 \times 10^{-3}$ M liposomal p-nitrophenyl stearate as a function of pH in buffered aqueous solutions with (o) and without (□) esterase at 37°C.
Figure 3.14: Percent p-nitrophenol released as a function of time due to hydrolysis of liposomal p-nitrophenyl stearate in esterase solutions at 37°C. The studies were conducted at pH 7.3 (•) pH 8.12 (○); pH 8.45 (□) and pH 9.48 (△).