Drug Stability in Liposomal Suspensions: Hydrolysis of Indomethacin, Cyclocytidine, and *p*-Nitrophenyl Acetate

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Abstract □ First-order rate constants (k_L) for hydrolysis of *p*-nitrophenyl acetate, cationic cyclocytidine, and anionic indomethacin in the presence of buffered liposomal suspensions of positive, negative, and neutral charge were compared to those determined in the corresponding buffers (k_B) using the ratio, $R_k = k_L/k_B$. Association between the reactants and the liposomes was evaluated by comparing assays for concentration in the filtrates (C_F) with the total concentration in the liposomal suspension (C_T) using $R_C = C_F/C_T$. Liposomes did not influence cyclocytidine hydrolysis rates and no association was observed ($R_k \simeq R_C \simeq 1$). In contrast, indomethacin showed ~80% reduction in hydrolysis rate and ~80% liposome association value ($R_k \simeq 0.2 \simeq R_C$). In neutral and negatively charged liposomal suspensions, *p*-nitrophenyl acetate displayed ~30% decrease in k_B ($R_k \simeq 0.7$) together with ~90% liposomal association ($R_C \simeq 0.1$). However, hydrolysis was greatly accelerated in positively charged liposomal suspensions. Loss was described by a biexponential equation where α is the fast and β is the slow pre-exponential coefficient and $\alpha/\beta/k_B$ = 39:6:1. The observed relationships between hydrolysis rates and reactant-liposome associations are reconciled in terms of the hydrophilicity of the reactants and the potential influence of the liposomes on the expected transition states for the hydrolysis reactions.

Keyphrases □ Indomethacin—hydrolysis, stability in liposomal suspensions, cyclocytidine, *p*-nitrophenyl acetate □ Stability—liposomal suspensions, hydrolysis of indomethacin, cyclocytidine, *p*-nitrophenyl acetate □ Hydrolysis—indomethacin, cyclocytidine, *p*-nitrophenyl acetate, stability in liposomal suspensions □ Liposomes, suspensions—drug stability, hydrolysis of indomethacin, cyclocytidine, *p*-nitrophenyl acetate

Interest in liposomes as potential drug delivery systems has prompted these investigations into the influence of liposomes on drug stability. This report compares the hydrolysis of a cation, an anion, and a neutral reactant in aqueous buffered solutions to that in buffered liposomal suspensions of positive, negative, and neutral charge.

BACKGROUND

The potential for changes in hydrolysis rates in micellar systems (1) and the implications to drug stabilization (2) are well documented and have been applied to aspirin (3–5) and indomethacin (6). Little has been reported on drug stability in liposomal suspensions although they are somewhat analogous to micellar systems. The rate of procaine hydrolysis was reduced in neutral liposomal suspensions relative to that in aqueous buffers, whereas 2-diethylaminoethyl-*p*-nitrobenzoate hydrolysis was accelerated at pH < 7.8 and retarded at pH > 7.8 (7). The degradation rate of *p*-nitrophenol acetate was accelerated by increasing liposomal lecithin concentration (8). These studies measured the total amount of drug in the suspension.

EXPERIMENTAL

Preparation of Liposomes—Cholesterol[1,2] (~7.4 mg) was placed in a 50-ml round bottom-stoppered flask together with either ~21 mg of dicetyl phosphate[1] (for negatively charged liposomes) or ~10.4 mg of stearylamine[1] (for positively charged liposomes). α-Tocopherol[1] (1 ml of 1.5×10^{-3} *M*) in chloroform (9, 10) was added, followed by 7 ml of chloroform and 1 ml of 1% lecithin in methanol–chloroform, 1:9[1]. The resulting solution was concentrated under vacuum for 3 min under a rotary evaporator at 42° (±2°). Evaporation was then continued at ambient temperature with the final traces of chloroform being removed by flushing with nitrogen.

Sixteen milliliters of buffer was added, and the mixture was agitated on a vortex mixer until the material from the sides of the flask formed a suspension of large liposomes. The flask was placed in a cup-horn sonicator[3] and cooled for 2 min using circulating water kept at 2°. The mixture was sonicated for 4 min at 60% power (maximum power = 20 kHz) using one-half second pulses per second. During the sonication the cooling water was kept circulating around the flask. Throughout the procedure the head space of the flask was periodically flushed with nitrogen to minimize oxidation. The size and shape of the liposomes were monitored using a microscope with an eye-piece micrometer together with particle size analysis (see *Kinetics of Hydrolysis*).

Analytical Methods—Stability-indicating spectrophotometric assays for indomethacin[4], *p*-nitrophenyl acetate, and cyclocytidine in buffered aqueous solutions, in total liposomal suspensions and in their filtrates are described below. The chromophoric hydrolysis products of cyclocytidine[1] (cytarabine[1]) and *p*-nitrophenol acetate[5] (*p*-nitrophenol[6]) were also measured. The products of indomethacin hydrolysis were nonchromophoric at 330 nm, which is near the 323-nm indomethacin absorption maximum.

To quench the hydrolysis of either indomethacin or *p*-nitrophenyl acetate in the total liposomal suspension, 1 ml of suspension was added to 4 ml of cold 0.5 *M* formic acid in water–methanol (2:8). The mixture was shaken and stored in a refrigerator. Prior to analysis it was warmed to room temperature and centrifuged at 3200 rpm for 30 min to remove liposomal material. Four milliliters of the supernatant solution was mixed with 4 ml of methanol and the resulting solution was centrifuged for 10 min. The UV absorbance of the resulting clear solution was determined at the appropriate wavelengths in a spectrophotometer[7], against a blank prepared in a similar manner omitting the reactant. The procedure for cyclocytidine was identical but employed 0.2 *M* HCl in water–methanol (1:9) to stabilize cytarabine, and the first centrifugation was 40 min.

Molar absorptivities (ϵ) were determined from the slopes of Beer's law plots using solutions in the buffers. The apparent molar absorptivities (ϵ') representing the procedure for assaying liposomal suspensions in the same buffers were similarly determined. Comparison of the values of ϵ' with those of ϵ indicated that the recovery was 93–100% as shown in Table I.

Since degradation products did not absorb at 330 nm, total indomethacin concentration, C_i, in the liposomal suspension was calculated from:

$$C_i = D\,(A_{330})/(\epsilon'_{330}) \qquad \text{(Eq. 1)}$$

where the dilution factor D is 10, ϵ'_{330} is the apparent molar absorptivity at 330 nm, and A_{330} is the absorbance of the final dilution at 330 nm.

Since the UV spectra of cyclocytidine and *p*-nitrophenyl acetate overlapped with those of their respective degradation products, the total absorbance of a mixture in the final dilution at a given wavelength, A_λ, is defined as:

$$A_\lambda = [\epsilon'_1(C_1) + \epsilon'_2(C_2)]/D \qquad \text{(Eq. 2)}$$

[1] Sigma Chemical Co., St. Louis, Mo.
[2] P-L Biochemicals, Milwaukee, Wis.
[3] Model W-375, Heat Systems—Ultrasonics, Plainview, N.Y.
[4] Merck, Sharp and Dohme Research Laboratories, West Point, Pa.
[5] Eastman Kodak Co., Rochester, N.Y.
[6] *p*-Nitrophenol was obtained by total hydrolysis of the ester in 0.1 *N* NaOH, 40°, for 5 hr.
[7] Model 250, Gilford Instruments, Oberlin, Ohio.

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Table I—Apparent Molar Absorptivities of Reference Standards Recovered from Liposomal Suspensions

λ(nm) x	λ(nm) y	Compound	n^a	$10^{-3}\epsilon'_{nx}$	$10^{-3}\epsilon'_{ny}$	Recovery[b], %
330	—	Indomethacin	1	6.40	—	~100
272	317	*p*-Nitrophenyl acetate	1	9.08	1.22	93
		p-Nitrophenol	2	3.20	11.60	94
260	285	Cyclocytidine	1	10.30	3.13	~100
		Cytarabine	2	4.70	14.00	~100

[a] $n = 1$ Designates reactant and $n = 2$ its hydrolysis product. [b] Average values from ϵ'_{nx} and ϵ'_{ny} where percent recovery = $100\ \epsilon'/\epsilon$.

Table II—Apparent First-Order Rate Constants for Hydrolysis in Buffers (k_B) and in Liposomal Suspensions (k_L,k_F), at 40°, and the Mean Diameters of Liposomes before (d_0) and after (d) Hydrolysis

Reactant[a]	Liposomal Charge	k in hr^{-1} k_B	k_L	k_F	R_C	μ d_0	d
Cyclocytidine	Positive	0.865[b]	0.858	0.786	1.12	2.38	2.30
	Negative	0.865[b]	0.789	0.756	1.18	2.21	2.26
	Neutral	0.865[b]	0.843	0.774	1.16	2.43	2.52
	Negative	0.121[c]	0.109	—	—	—	—
Indomethacin	Positive	0.346[d]	0.054	—	~0.10[e]	2.10	2.26
	Negative	0.346[d]	0.110	0.106	0.28	2.17	2.17
	Neutral	0.346[d]	0.0783	—	~0.20[e]	2.38	2.52
p-Nitrophenyl-acetate	Negative	0.362[c]	0.249	0.300	0.086	2.10	2.34
	Neutral	0.362[c]	0.268	0.300	0.144	2.14	2.38
	Positive	0.362[c]	$\frac{\alpha}{14.0}$	$\frac{\beta}{2.05}$	0.508[f]	2.14	2.21

[a] $C_0 \simeq 9 \times 10^{-4}$ *M*. [b] Borate buffer: 0.320 *M* H_3BO_3; 0.140 *M* NaH_2BO_3; 0.350 *M* NaCl; pH 8.31. [c] Phosphate buffer: 0.0140 *M* NaH_2PO_4; 0.100 *M* Na_2HPO_4; 0.086 *M* NaCl; pH 7.51. [d] Carbonate buffer: 0.135 *M* $NaHCO_3$; 0.065 *M* Na_2CO_3; 0.075 *M* NaCl; pH 0.37. [e] Filtrates were not concentrated enough to obtain more accurate estimates. [f] Average value during the beta phase.

where $D = 10$, C_1 and C_2 are the total concentrations of reactants and products, respectively, in the liposomal suspensions, and ϵ'_1 and ϵ'_2 are their apparent molar absorptivities at wavelength λ. The total absorbance determined at each of two wavelengths, A_x and A_y, then results in two simultaneous equations which may be solved to yield:

$$C_1 = D[\Delta_{1x}(A_x) - \Delta_{1y}(A_y)] \quad \text{(Eq. 3)}$$

$$C_2 = D[\Delta_{2x}(A_x) - \Delta_{2y}(A_y)] \quad \text{(Eq. 4)}$$

where $\Delta_{1x} = \epsilon'_{2y}/(\epsilon'_{1x}\epsilon'_{2y} - \epsilon'_{1y}\epsilon'_{2x})$, $\Delta_{1y} = \epsilon'_{2x}/(\epsilon'_{1x}\epsilon'_{2y} - \epsilon'_{1y}\epsilon'_{2x})$, $\Delta_{2x} = \epsilon'_{1y}/(\epsilon'_{2x}\epsilon'_{1y} - \epsilon'_{1x}\epsilon'_{2y})$, $\Delta_{2y} = \epsilon'_{1x}/(\epsilon'_{2x}\epsilon'_{1y} - \epsilon'_{1x}\epsilon'_{2y})$, ϵ'_{1x} and ϵ'_{1y} are the apparent molar absorptivities of the reactants, and ϵ'_{2x} and ϵ'_{2y} are those of the products, at wavelengths x and y, respectively (Table I).

For the analysis of filtrates, 2-ml aliquots of reactions in the presence of liposomal suspensions were gently filtered using disposable cartridges[8] and a syringe. In the case of indomethacin and *p*-nitrophenyl acetate, 0.5 ml of the filtrate was mixed with 2 ml of cold 0.5 *M* formic acid in water–methanol (2:8). The procedure for cyclocytidine was identical, except that 0.2 *M* HCl in water–methanol (1:9) was employed instead of 0.5 *M* formic acid. Absorbances were measured in microcuvetes and the concentrations were determined using the ϵ values in Table I in Eqs. 1, 3, and 4 with $D = 5$. Total particle counts[9] which were 4×10^6/ml in the liposomal suspensions were reduced to background (<2000/ml) in the filtrate by this procedure.

Kinetics of Hydrolysis in Buffers and in Buffered Liposomal Suspensions—Rates of hydrolysis of cyclocytidine, indomethacin, and *p*-nitrophenyl acetate were studied at 40° in the aqueous buffers described in Table II. Aliquots were removed as a function of time and analyzed for concentrations.

Liposomal suspensions (Table II) were prepared as described and heated at 40° for 12 hr with constant agitation[10]. An aliquot of a stock solution of the reactant was then added to 12 ml of the liposomal suspension, which was maintained at 40° with continued agitation. At appropriate intervals aliquots of the mixture were removed, the reaction head-space flushed with nitrogen, and the samples were analyzed for total concentration in the liposomal suspension and also in the filtrate. Before, and after the reaction, 6-μl aliquots of the liposomal suspension were diluted with 10 ml of the same buffer in which they were prepared. One milliliter of this dilution was further diluted with 10 ml of buffer, and the particle size of the liposomes in this suspension was determined[9].

Hydrolysis rates in filtrates in the absence of liposomes were shown to be the same as those in their corresponding buffered solutions by using the following procedure. Sixteen milliliters of positively charged liposomal suspension was prepared using carbonate buffer and heated at 40° for 12 hr with constant agitation[10]. The suspension was centrifuged at 2600 rpm for 50 min, and the bulk of the liposomes that concentrated at the top of the mixture were removed[11]. Five milliliters of the remaining suspension was filtered through several disposable filter cartridges[8]. The filtrate was used to study the hydrolysis of indomethacin as described previously for aqueous buffers at 40°. This procedure was also employed to study the hydrolysis of *p*-nitrophenyl acetate in the filtrate obtained from positively charged liposomes prepared with phosphate buffer. No difference was observed between the rate constants in buffer and in the corresponding filtrate. These two reactions were chosen because they had shown the greatest change in rate constants in the presence of liposomes.

To estimate the maximum volume occupied by the liposomes in a suspension, 10 ml of a negatively charged suspension prepared in carbonate buffer was heated at 40° for 12 hr with constant agitation. The suspension was then heated for an additional 24 hr with no agitation. The liposomes that concentrated at the top were removed, and the volume of the remaining mixture was determined to be 9.2 ml.

RESULTS

Apparent First-Order Hydrolysis—Except for the loss of *p*-nitrophenyl acetate in the presence of positive liposomes (to be discussed), all kinetic data were adequately described by the first-order equation,

$$\ln C_t = \ln C_0 - k_{obs}t \quad \text{(Eq. 5)}$$

where t is time, C_0 and C_t are the reactant concentrations initially and at time t, and k_{obs} is the observed first-order rate constant.

Cyclocytidine—The sum of the cyclocytidine and cytarabine concentrations as a function of time equaled the initial concentration of cyclocytidine. If the ratio of the rate constant in the presence of liposome

[8] Millex-GS 0.22 μm Filter unit, Millipore Corp., Bedford, Mass.
[9] Elzone Model 80 XY, Particle Data Inc., Elmhurst, Ill.
[10] Submersion Rotator, Scientific Industries, Inc., Queens Village, N.Y.
[11] In these concentrated buffers, liposomes ascended on centrifugation. The direction was reversed if buffers were diluted tenfold before centrifuging.

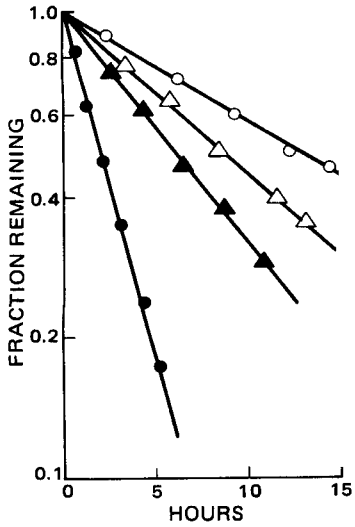


Figure 1—*First-order plot for fraction of indomethacin remaining in carbonate buffer, pH 9.37, (●) and in the presence of liposomes of negative (▲), neutral (△), or positive (○) charge at 40°.*

(k_L) to that in buffered solution (k_B) is defined as:

$$R_k = k_L/k_B \qquad \text{(Eq. 6)}$$

it is apparent that $R_k \simeq 1$ (Table II).

The filtrate concentration (C_F) can be compared with the corresponding concentration in the total liposomal suspension (C_T) using the ratio, R_C where:

$$R_C = [C_F/C_T]_{t_{0.5}} \qquad \text{(Eq. 7)}$$

Although the differences between C_F and C_T are small, the filtrate concentrations were consistently higher than the liposomal concentrations giving R_C values >1 (Table II). This may be due to a small decrease in volume by removal of the liposomes.

Indomethacin—The reduced rates of indomethacin hydrolysis in various liposomal suspensions as compared with that in liposome-free buffer are shown in Fig. 1. This reduction is reflected in the R_k values which are 0.156, 0.318, and 0.226 in positively charged, negatively charged, and neutral liposomal suspensions, respectively.

In negatively charged liposomes R_C = 0.28, and the first-order rate constant obtained from the filtrate data was k_F = 0.106 hr^{-1} (Fig. 2) which agrees with its corresponding k_L value. In filtrates from the neutral and positively charged liposomal suspensions, the low concentrations of indomethacin (relative to assay sensitivity) prevented the accurate estimation of first-order rate constants, k_F. However, average values for R_C could be estimated from the ratios of measured C_F to calculated C_T values. Since each ratio is comprised of a small numerator (C_F) and a large denominator (C_T), the variability in R_C is minimized (Fig. 3, Table II).

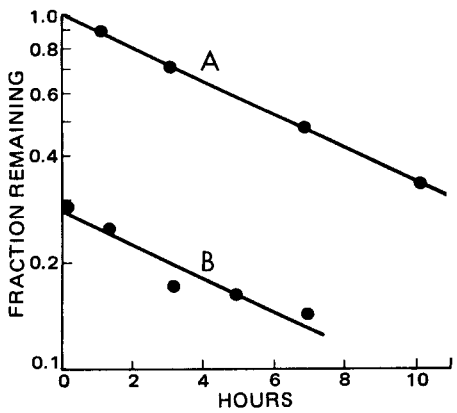


Figure 2—*Comparison of first-order plots for total indomethacin in a negatively charged liposomal suspension (A) to the fraction in the filtrates (B) at 40° in carbonate buffer, pH 9.37.*

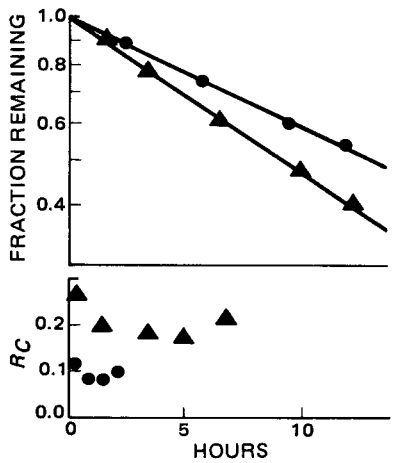


Figure 3—*Calculated R_C values for indomethacin as a function of time and their corresponding first-order plots in neutral (▲) and positively charged (●) liposomal suspensions at 40° in carbonate buffer, pH 9.37.*

p-Nitrophenyl Acetate—The sum of *p*-nitrophenyl acetate and *p*-nitrophenol concentrations as a function of time equaled the initial concentration of *p*-nitrophenyl acetate. The rate of loss of *p*-nitrophenyl acetate in phosphate buffer was decreased in neutral and negatively charged liposomal suspensions. This reduction is reflected in the R_k values of 0.740 and 0.688, respectively. The values of R_C are 0.144 and 0.086 for neutral and negatively charged liposomal suspensions respectively, and the first-order rate constants in the filtrates (k_F) are close to their respective k_L values (Table II).

Semilogarithmic plots of the concentration of *p*-nitrophenyl acetate in positively charged liposomal suspensions as a function of time showed a rapid initial decrease followed by a much slower loss. The biphasic curves were analyzed by the following equation:

$$F = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 8)}$$

where $F = C_t/C_0$, and $\alpha > \beta$ (Fig. 4). Nonlinear regression analyses provided average values of A = 0.406, B = 0.595, α = 0.233 min^{-1}, and β = 0.0341 min^{-1}. Experimental C_F values and calculated C_T values determined during the beta phase were used to calculate an average R_C value, of 0.508.

DISCUSSION

Cyclocytidine, indomethacin, or *p*-nitrophenyl acetate, in liposomal suspensions can hydrolyze in the bulk aqueous solution or in the liposomal phase. The association of reactant with the liposomal phase might occur in several ways. Those interactions that bring the compounds into the proximity of an organic environment can cause a change in the hydrolysis rate in accordance with the expected solvent effects (Fig. 5).

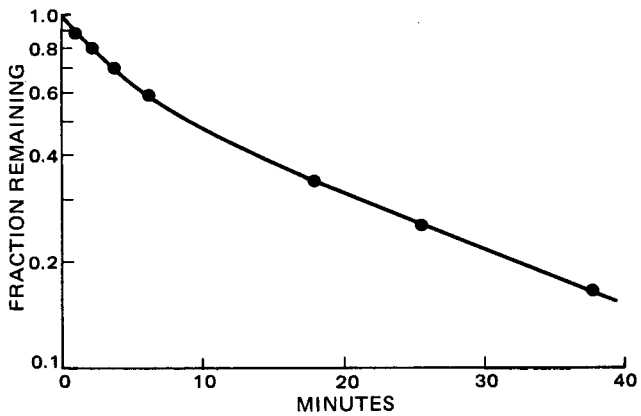


Figure 4—*Semilogarithmic plot for fraction of* p*-nitrophenyl acetate remaining as a function of time in positively charged liposomal suspension at 40° in phosphate buffer pH 7.51. Curve is drawn from nonlinear regression analysis based on Eq. 8.*

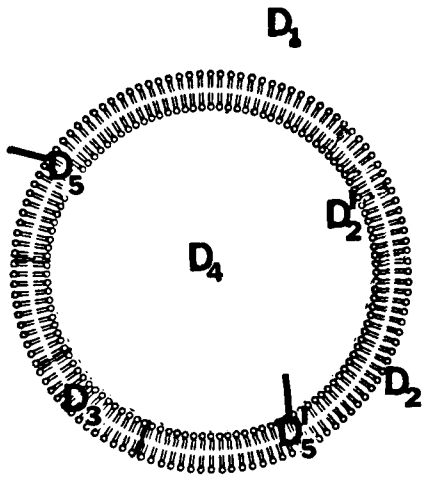


Figure 5—*Possible locations of a compound,* D, *in a liposomal suspension;* D_1 *is in the external aqueous phase;* D_2 *and* D'_2 *are associated with the outer and inner walls;* D_3 *is associated with the hydrocarbon portion of the lipid bilayers;* D_4 *is dissolved in the aqueous portions of the liposome; and* D_5 *and* D'_5 *are solubilized in the hydrocarbon portion of the lipid bilayer with portions protruding into the aqueous environments.*

Media with low ion-solvating power will inhibit the creation or concentration of charges while accelerating charge destruction (11). A change in rate would, therefore, depend on the reaction mechanism and the species involved.

The hydrolysis of cyclocytidine was not affected by the presence of liposomes as evidenced by the similarity between the values of the first-order hydrolysis rate constants determined in aqueous buffer (k_B), in liposomal suspensions (k_L), and using the data representing the filtered samples obtained from the reactions in suspensions (k_F, Table II). Assay of the filtrates accounted for the total drug in the liposomal suspensions. Cyclocytidine, a highly polar, water soluble, positively charged compound, probably resists association due to its hydrophilicity. This, in itself, would not preclude surface participation in the reaction rate. Positive and negative liposomal surfaces could affect reaction intermediates and/or transition states, in that positively charged liposomes would be expected to stabilize negatively charged species and *vice-versa*. Since hydroxyl anion attack on the cyclocytidine cation produces a neutral transition state, no sensitivity to surface charge would be anticipated.

In contrast, indomethacin in the presence of positively charged, negatively charged, and neutral liposomes, and *p*-nitrophenyl acetate in negatively charged and neutral liposomal suspensions all provide hydrolysis rate constants (k_L) that are slower than those in aqueous buffer (k_B). Since this loss can be described by the first-order equation:

$$\frac{-d(DT)}{dt} = k_L(DT) \qquad \text{(Eq. 9)}$$

where (DT) is the total amount of drug in the liposomal suspension at time t, and k_L is the apparent first-order rate constant for loss of drug, the simplest kinetic scheme (Scheme I) that can represent this data is:

$$DF \underset{}{\overset{\text{Fast}}{\rightleftharpoons}} DL$$
$$\downarrow k_B \qquad\qquad \downarrow k_\Delta$$

Scheme I

where (DF) is the drug in the bulk aqueous phase, (DL) is the drug associated with the liposomes and, k_B and k_Δ are the rate constants for the loss of drug from the bulk aqueous and liposomal phases, respectively. It is obvious that:

$$k_L(DT) = k_B(DF) + k_\Delta(DL) \qquad \text{(Eq. 10)}$$

and, therefore, the rate constant k_L can be described:

$$k_L = k_B f_B + k_\Delta k_L \qquad \text{(Eq. 11)}$$

where $f_B = (DF)/(DT)$, and $f_L = (DL)/(DT)$. A similar expression has been applied to the hydrolysis of a number of compounds in the presence of micelles (1). Since the volume occupied by the liposomes in these suspensions was found to be <10%, the ratio $R_C = [C_F]/[C_T]$ is approximately equal to $f_B = (DF)/(DT)$. Setting $f_L = (1 - f_B)$ and substituting R_C for f_B in Eq. 11 results in:

$$k_L = k_B(R_C) + k_\Delta(1 - R_C) \qquad \text{(Eq. 12)}$$

The rate constants for the hydrolysis of indomethacin in positively charged, negatively charged, and neutral liposomes (k_L) are 15.6, 31.8, and 22.6% of k_B. Indomethacin is associated with the liposomes to the extent of 90, 72, and 80% in positively charged, negatively charged, and neutral liposomes, respectively (R_C values, Table II). The extent of this association could be a function of electrostatic attraction. The negatively charged indomethacin molecules would be attracted to the positively charged liposomes to the greatest extent followed by the neutral liposomes, while the carboxylate anion would be repelled by the negatively charged liposomes. At the reaction pH, indomethacin is made up of a hydrophilic carboxylate anion and the remaining hydrophobic moiety. Thus, if indomethacin associates with the liposomes, it could be positioned at the interface of the aqueous bulk and the hydrocarbon portion of the lipid bilayer as shown in Fig. 5.

A similar effect has been reported for the hydrolysis of the sodium salt of mono-*p*-nitrophenyl dodecanedioate in micellar aggregates (12). By analogy to that study, the carboxylate anion of indomethacin could be in an aqueous environment, while the hydrophobic part containing the amide linkage could be stabilized in the hydrocarbon environment of the liposomes. In addition, the hydrolysis of indomethacin involves the attack of a hydroxide ion on the negatively charged molecule, thus causing the collection of similar charges in the transition state. This process would be inhibited by the bilipid layer.

The first-order rate constants for hydrolysis of *p*-nitrophenyl acetate in the presence of negatively charged and neutral liposomes are 68.8% and 74.0%, respectively, of that in the aqueous buffer. *p*-Nitrophenyl acetate is ~90% associated (Table II). Unlike indomethacin, *p*-nitrophenyl acetate does not have a formal charge. If *p*-nitrophenyl acetate associates with the liposomes, it would not selectively shield the ester linkage away from the aqueous environment. It has been shown in micellar phases, that compounds having a slight polar nature (such as nitrobenzene) are solubilized at the surface of a micelle rather than in the hydrocarbon interior (13, 14). Consequently, *p*-nitrophenyl acetate could be largely exposed to the aqueous environments both within the liposomes and at the bulk aqueous interface. This could account for the fact that ~90% association resulted in only an ~30% reduction in rate constant. Since hydroxide attack on *p*-nitrophenyl acetate would provide a negative transition state, electrostatic depression in hydrolysis rate would apply.

The decrease of *p*-nitrophenyl acetate concentration in the presence of positively charged liposomes is described by a biexponential equation in which the exponential coefficients, α and β, are 38.6 and 5.65 times faster, respectively, than k_B. This acceleration and biexponential hydrolysis rate might be attributed to elecrostatic stabilization of the negatively charged transition state by the positive charges on the surface of the liposomes in accordance with the following kinetic scheme (Scheme II):

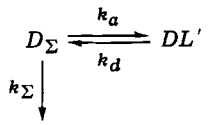
$$DF \underset{}{\overset{\text{Fast}}{\rightleftharpoons}} DS \underset{k_d}{\overset{k_a}{\rightleftharpoons}} (DL')$$
$$\downarrow k_B \qquad \downarrow k_{cat} \qquad\qquad \downarrow k_\Delta$$

Scheme II

Table III—Percent Comparisons for Hydrolysis in Liposomal Suspension Relative to the Buffer ($100\ k_L/k_B$), Hydrolysis of Associated Reactant Relative to Free ($100\ k_\Delta/k_B$), Percentage of Reaction in the Associated Fraction ($100\ k_\Delta f_L/k_L$), and the Percent Associated ($100\ DL/DT$)

Reactant	Liposomal Charge	% = (100) Numerator/Denominator k_L/k_B	k_Δ/k_B	$k_\Delta f_L/k_L$	DL/DT
Cyclocytidine	(+ − 0)	95 ± 4	0	0	0
Indomethacin	(+)	16	6	36	90
	(−)	32	5	12	72
	(0)	23	3	12	80
p-Nitrophenyl acetate	(−)	69	66	88	91
	(0)	74	70	81	86

Surface catalysis has been suggested for the enhanced rate of hydrolysis of *p*-nitrophenyl hexanoate in solutions containing cationic micelles (15). Here, (*DF*) is in rapid pseudo equilibrium with (*DS*), the ester associated with the exterior surface (Fig. 4), (*DL'*) represents liposome–ester association by the remaining mechanisms, k_{cat} is the rate constant for hydrolysis of *p*-nitrophenyl acetate catalyzed by the positively charged liposomal surface, and k_a and k_d are the rate constants for the association and dissociation of (*DS*) with (*DL'*). Since the ratio $\alpha/\beta/k_B$ is 39:6:1 and R_C (beta phase) is ~0.5, it follows that $k_{cat} \gg k_B > k_\Delta$ and the simplest representation would be to set $(D_\Sigma) = (DF + DS)$ and $k_\Sigma = (k_B + k_{cat})$ allowing (Scheme III):



Scheme III

where k_Δ is considered insignificant relative to k_Σ. This is analogous to those cases discussed under Scheme I wherein liposome-associated reactant was stabilized relative to that in liposome-free buffer. Biexponential equations and the calculations for their individual rate constants in models described by Scheme III have been published (16). The fraction remaining, $F = (DT)/(DT)_0$, is given by:

$$F = \left(\frac{\alpha - k_a - k_d}{\alpha - \beta}\right) e^{-\alpha t} + \left(\frac{k_a + k_d - \beta}{\alpha - \beta}\right) e^{-\beta t} \qquad \text{(Eq. 13)}$$

which describes data of the shape shown in Fig. 4. Under these conditions, $k_\Sigma = A\alpha + B\beta = 6.9\ hr^{-1}$, $k_d = \alpha\beta/k_\Sigma = 4.2\ hr^{-1}$, and $k_a = \alpha + \beta - k_\Sigma - k_d = 5.0\ hr^{-}$. The phase ratio, $(DL')/(D_\Sigma)$ may be calculated from $k_a/(k_d - \beta) = 2.3$ (17) which can be used to estimate $R_C = C_F/C_T \simeq 0.3$. Although Scheme III is an oversimplification, the estimated rate constants are reasonable, the shape of Fig. 4 is consistent with Eq. 8, and the calculated R_C approaches that observed (R_C = 0.5, Table II).

Comparisons—While the net influence of liposomes on reaction rates is reflected by the k_L/k_B ratio (R_k, Eq. 6), an overall assessment requires concomitant examination of several aspects. The reactivity in the associated phase (*DL*) relative to that in the bulk aqueous phase (*DF*) is given by k_Δ/k_B, but the percentage contribution of each phase to the reaction (k_L) is 100 $k_\Delta f_L/k_L$ and 100 $k_B f_B/k_L$ rather than the percent of liposomal association (100 *DL/DT*) or the rate constants themselves. It is the competition between $k_\Delta f_L$ and $k_B f_B$ that controls the degree to which the overall process occurs in each phase. Collectively, the four parameters listed in Table III provide an aggregate interpretation of the total kinetic behavior, whereas any one of the components taken alone would be inadequate.

Although differences in pH preclude absolute comparisons of the three reactants, some outstanding contrasts can be noted (Table III). Liposomal association of cyclocytidine was not detected, and its hydrolysis rate constants in all four suspensions are approximately equal to that in the buffer. Both reactant distribution and hydrolysis appear limited to the aqueous bulk. In contrast, *p*-nitrophenyl acetate (in the presence of negative and neutral liposomes) shows 81–88% of the reaction occurring in the *DL* phase. This is the consequence of the percentage values being $k_L/k_B \approx k_\Delta/k_B \approx 70\%$ with 90% association. Rate constants for hydrolysis in the liposomal phases (k_Δ values in hr^{-1} calculated from Eq. 12) are 0.252 in neutral and 0.238 negatively charged liposomal suspensions. Thus, $k_B \approx 1.4k_\Delta$, but only 10% remains in the *DF* phase. Hydrolysis occurs primarily in the *DL* phase at a slightly reduced rate. This is consistent with the hypothesis that the associated ester remains largely accessible to the aqueous environment in the liposome.

Indomethacin is also highly associated (72–90%), but the values of k_Δ were ~0.02, 0.018, and ~0.01 hr^{-1} in positively charged, negatively charged, and neutral liposomes, respectively. In this case the percent of $k_L/k_B \approx 24\%$ since $k_\Delta/k_B \approx 5\%$. The high degree of association subjects most of the reactant to a greatly reduced rate constant. Only 12–36% of the process occurs in the *DL* phase. This provides a significant depression in the overall hydrolysis rate constant, but the reaction occurs primarily in the aqueous bulk. This reduced reactivity in the *DL* phase implies protection of the reactive center by liposomal association.

Thus, Table III provides examples of three different situations. In one case, cyclocytidine is limited to the aqueous buffer and no liposomal influence is observed. The remaining two cases are predominantly associated in roughly equivalent percentages but with differing consequences. Indomethacin reactivity is greatly reduced with only a minor contribution to the reaction occurring in the associated phase. *p*-Nitrophenyl acetate reactivity is slightly reduced occurring primarily in the associated phase where the rate constant is 70% of the constant for the aqueous bulk. The exception in this study is *p*-nitrophenyl acetate in positively charged liposomal suspensions which serve as catalysts to the ester hydrolysis as discussed in the previous section.

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