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STABILIZATION OF LOCAL ANESTHETICS IN LIPOSOMES

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The first-order hydrolysis rate constants of local anesthetic drugs have been measured in aqueous buffer solution, k_B, and in liposomes, k_{obs}, at pH 12.2 and 30°C. Also, the fraction of drug associated with the lipid phase, f_L, and the partition coefficient between the aqueous and lipid phase, K^L_B, were determined. The stability of benzocaine was measured in several liposome compositions, and as a function of benzocaine concentration, phospholipid concentration, and ionic strength of the medium. Values of the rate constant in the lipid phase, k_L, were estimated from a simple kinetic model and predictions of the relative contributions of k_L and k_B to k_{obs} were made. Different methods of liposome preparation, and the stabilities of local anesthetics in liposomal and micellar systems were compared.

INTRODUCTION

Liposomes are colloidal dispersions of lipid molecules arranged as bimolecular leaflets in an approximately spherical shape and enclosing compartments of aqueous medium. These easily prepared formulations have created considerable interest in studies of drug-membrane interactions[1] and as drug delivery systems[2]. Recently, an interesting potential application of liposomes to stabilize drugs has been given some attention[3-6]. These attempts have been mainly aimed at incorporating drug into phospholipid bilayers by equilibrium partitioning with the expectation that the hydrocarbon environment of the bilayer offers protection against catalytic hydrolysis in a manner similar to that found in micellar systems[7-9]. Depending on the composition of the liposomes and the chemical nature of the solute increases in stability have ranged from 0% (e.g.cyclocytidine[5]) to about 90% for a trimethylammonium halide[6]. These results indicate that the important factors involved in the stabilization of esters or amides in liposomes are the depth of the reactive centre of the solute molecule in the phospholipid bilayer membrane[6] and the fraction of the total drug associated[5] with or partitioned[3] in the liposomes. There is also evidence, however, that drug bound to sites in the bilayers may also give rise to improved stability[10] whereas ester adsorbed or bound to the liposome surfaces can accelerate its loss in comparison to its concentration in aqueous buffer solution[11].

Local anesthetic drugs are esters whose hydrolysis kinetics have been studied in aqueous solution[12,13]

and micelles[14-16]. Procaine has also been stabilized in neutral, fluid liposomes[3] and in lyotropic smectic mesophases[17]. A study of a series of these agents in liposome systems under various conditions offers a convenient means of determining the importance of the role of partitioning on stability in this type of formulation.

MATERIALS

The lipids used as received in this study were supplied by Sigma Chemical Co., St. Louis, Missouri and included: L-α - dimyristoylphosphatidylcholine, 98% (DMPC), L-α -dipalmitoylphosphatidylcholine, 99%(DPPC), L-α -phosphatidylcholine, type V-E from egg yolk(EPC), sphingomyelin from bovine brain(SPHING), phosphatidyl-serine from bovine brain, 98%(PS), and cholesterol, 99%(CHOL). Local anesthetics included benzocaine, U.S.P.(J.T. Baker Chemical Co.), procaine hydrochloride, U.S.P.(Allen & Hanbury), tetracaine hydrochloride(Sigma Chemical Co.), methyl p-amino-benzoate, 97%, n-propyl p-aminobenzoate, 99%, and n-butyl p-aminobenzoate, 97%(Pfaltz & Bauer). Indomethacin(Sigma Chemical Co.) was also studied for comparison. The degradation products, p-aminobenzoic acid(Allen & Hanbury) and 4-(butylamino) benzoic acid, 98%(Aldrich Chemical Co.) were required in the analytical procedure. All other chemicals and solvents were reagent grade and water was glass-distilled.

METHODS

Aqueous buffer solutions used in these studies had the following compositions: .059M KH_2PO_4+.007M

Na_2HPO_4(pH 6.0),.013M KH_2PO_4+.054M Na_2HPO_4 (pH 7.4), .06M boric acid + .14M Na_2CO_3(pH 10.0), and .045M NaOH(pH 12.2). The ionic strength was adjusted with NaCl usually to 0.15 and checked using an osmometer (Model 3D, Advanced osmometer, Advanced Instruments, Mass.)

Liposome Preparation

A stock multilamellar liposome preparation was made as previously described [18]. Briefly, a film of phospholipid (57 micromoles) was formed on the inside wall of a 1L round-bottom flask by first dissolving the phospholipid in chloroform then removing the solvent by rotary evaporation at 40°. It was subsequently flushed with N_2 gas then placed in a vacuum oven at 40° to dry over P_2O_5 for 12-14 hr to remove the last traces of solvent. Liposomes were formed by adding 10 ml of aqueous buffer solution warmed to at least 10° above the phase transition temperature of the phospholipid then vortex-mixed for 10 min. Drugs were incorporated into liposomes by mixing equal volumes of liposomes and aqueous buffer solution of drug and thoroughly mixing. Some experiments were conducted in which the drug was initially incorporated in liposomes via the organic phase. Thus, procaine was added along with the phospholipid (51 micromoles) to chloroform and a film produced in the usual manner. Liposomes were then formed after the addition of aqueous buffer solution (20 ml) as before.

Kinetic Studies and Analysis

The stabilities of the local anesthetics were determined in aqueous buffer solution and in liposomes

at pH 12.2 and 30°. Similar conditions have been used by others[14] in stability studies of benzocaine in micellar solutions because of the convenient rate of hydrolysis under these conditions and the constancy of the OH^- concentration during the hydrolysis reactions.The liposome preparation was divided into 2ml quantities, placed in 25ml stoppered volumetric flasks, then maintained at constant temperature in a shaking water-bath (Dubnoff metabolic shaker, Precision Scientific Co.). A test sample was diluted to volume with isopropyl alcohol after a designated time interval resulting in a clear solution then assayed spectrophotometrically at λ_{max} (uv/vis spectrophotometer, Model 25, Beckman Instruments, Inc. CA).

Since the degradation product of the local anesthetics caused interference in the analysis, the total absorbance (A_λ) at λ_{max} of the local anesthetic in the system is given by

$$A_\lambda = \varepsilon C + \varepsilon_1 C_1 \qquad (1)$$

where C and C_1 are the concentrations of reactants and degradation products, respectively, and ε and ε_1 are their corresponding molar absorptivities at λ_{max}. The fraction of drug remaining is determined at any given time from Eq.(2):

$$C/C_0 = \frac{A_\lambda/C_0 - \varepsilon_1}{\Delta\varepsilon} \qquad (2)$$

where C_0 is the initial concentration of drug, and C is its concentration at time, t, and $\Delta\varepsilon = \varepsilon - \varepsilon_1$. The values of ε and ε_1 at their respective λ_{max} are given in Table 1.

TABLE 1

Structures and Spectral Characteristics of Local Anesthetics and Their Degradation Products:

Local Anesthetic	Structure[a]	λ(nm)	Molar Absorptivity
Benzocaine	X=C_3H_7, Y=H	291	21,552
Procaine	X=C_2H_4-N(C_2H_5)(C_2H_5)	293	22,747
	Y=H		
Tetracaine	X=C_2H_4-N(CH_3)(CH_3)	309	29,552
	Y=C_4H_9		
Methyl p-amino benzoate	x=CH_3, Y=H	294	18,934
n-Propyl p-amino benzoate	X=C3H7, Y=H	294	19,235
n-Butyl p-amino benzoate	X=C_4H_9, Y=H	294	19,156
p-Aminobenzoic acid[b]	X=H, Y=H	291	5,721
		293	4,946
		294	4,944
p-Butylamino-benzoic acid[c]	X=H, Y=C_4H_9	309	5,437

[a] general structure: Y-HN-C$_6$H$_4$-CO-O-X

[b] degradation product of all local anesthetics except tetracaine.

[c] degradation product of tetracaine.

The fraction of drug associated with the phospholipid phase (f_L) at the beginning of the kinetic studies was determined from the difference between the total amount of drug in the liposomal suspension (D_T) and the residual amount of drug in the supernatant (D_B) after centrifugation (135,000 xg, 30 min, 30°; L8-55 ultracentrifuge, Beckman Instruments Inc. CA) divided by D_T.

Partition Coefficients in Liposomes

Liposomes, which were freshly prepared in aqueous buffer at a lower pH to minimize degradation but maintaining the drug in a completely unionized state (except indomethacin), were equilibrated at 30° for 30 min. then centrifuged as before at the same temperature at 135,000 xg for 30 min. Subsequently, the supernatant was carefully withdrawn by Pasteur pipette, diluted with isopropyl alcohol and analyzed spectrophotometrically. Concentrations of drug in the supernatants, C_B, were determined from a calibration curve and the residual amounts in the phospholipid phase were calculated from the mass balance. The molal partition coefficients, K^L_B, were determined from Eq.(3):

$$K^L_B = \frac{(C_T - C_B)\, w_1}{C_B \cdot w_2} \qquad (3)$$

where C_T is the total concentration of drug (mg/ml) in the system, w_1 and w_2 are the weights of the aqueous and phospholipid phases, respectively.

All kinetic and partition coefficient experiments were performed at least in triplicate and the results averaged.

RESULTS

The hydrolysis of the local anesthetics in aqueous buffer solution or in liposome preparations obeyed apparent first-order kinetics for at least three half-lives as shown in Fig. 1 and is given by Eq.(4):

$$C/C_0 = \varepsilon^{-kt} \quad (4)$$

where C_o and C are the molar concentrations of drug initially and at time t, respectively, and k is the pseudo first-order hydrolysis rate constant. For hydrolysis in aqueous buffer solution $k = k_B$ and in liposome systems $k = k_{obs}$. Hydrolysis in the lipid phase (k_L) and in the external aqueous phase of liposomes (k_b) was taken to obey Eq.(4) and that $k_b = k_B$[5]. Thus,

$$k_{obs} = k_L f_L + k_B f_B \quad (5)$$

where f_L and f_B are the fraction of drug in the lipid and aqueous phases, respectively.

Determination of the relative improvement in the stabilities of liposomes of various compositions was made from the results of k_{obs} and k_B expressed as

$$\% \text{ increase in stability} = \frac{k_B - k_{obs}}{k_B} \times 100 \quad (6)$$

Table 2 indicates a significant variation in the stabilization of benzocaine (a 4-fold change) by selecting different phospholipids or

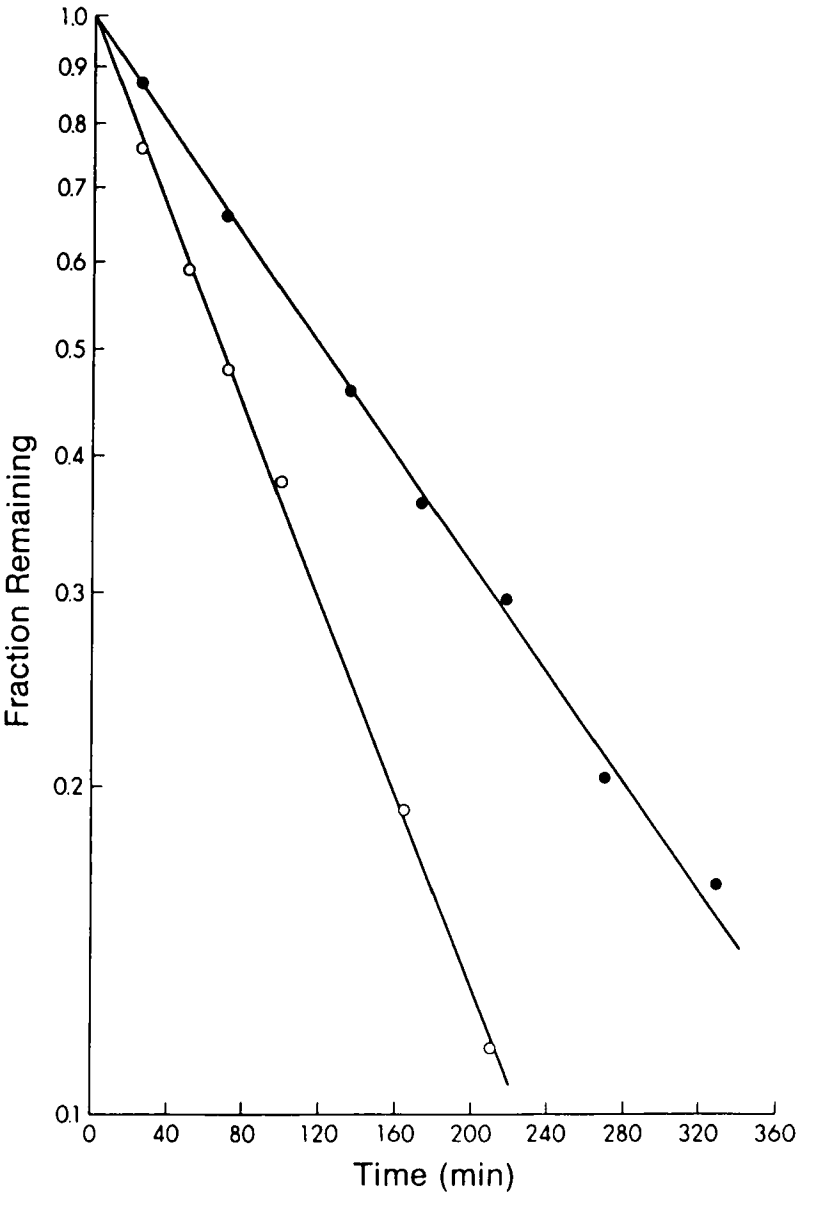


FIGURE 1

First-order hydrolysis kinetics of benzocaine in aqueous buffer solution, ○ and in liposomes, ● at pH 12.2(.045M NaOH) and 30°C. The initial benzocaine concentration was 0.76mM and the phospholipid(DMPC) concentration was 5.8mM.

TABLE 2

The Stabilization of Benzocaine in Liposomes of Various Compositions at pH 12.2 and 30°C.[a]

Liposome Composition[b]	% Increase in Stability[c]
PS	47
EPC	39
DMPC	34
DPPC	23
SPHING	22
DMPC:CHOL (3:1)	25
DMPC:CHOL (1:1)	12

[a]the initial benzocaine concentration was 0.76mM; the total lipid concentration was 2.9mM.
[b]see text for definition of abbreviations; mole ratios are shown in brackets.
[c]using Eq.(6).

phospholipid:cholesterol combinations. Neutral liposomes of phosphatidylcholines, which exist in a fluid liquid crystalline state (EPC, DMPC) at the temperature of the experiment (30°), protects benzocaine against hydrolysis to the extent of 35-40%. In contrast, DPPC is in a more rigid gel state at this temperature and the stability is increased by only 23%. Sphingomyelin liposomes, although in a fluid state, allow benzocaine to be stabilized only to the extent of 22%. On the other hand, negatively-charged liposomes of PS yield the greatest protection against hydrolysis

under the present conditions. The addition of CHOL to DMPC liposomes causes k_{obs} to increase and at a 1:1 mole ratio only a 12% increase in the stability of benzocaine was obtained. Thus, the addition of 50% CHOL to the liposome composition results in about a 3-fold decrease in effectiveness of the phospholipid (DMPC) liposomes to stabilize benzocaine.

Studies were conducted to determine whether variation of the content of benzocaine in liposomes or the osmolarity of the aqueous medium produced significant change in the kinetics of degradation. The results in Fig. 2 show a parallel decrease in k_{obs} with increasing benzocaine concentration in liposomes of DMPC or DPPC. However, a 4-fold increase in benzocaine concentration resulted in only a 15% decrease in k_{obs}. Similarly, ionic strengths of the aqueous medium ranging from 0.05 to 0.25 had little effect on k_{obs} (Table 3) even though the liposomes exist in various swelling states under these conditions[19].

The variation of k_{obs} of benzocaine, procaine and tetracaine in DMPC or DPPC liposomes as a function of the phospholipid concentration over the range 1.4 - 28.7mM is illustrated in Fig.3. Each curve is characterized by an initial rapid decrease in k_{obs} followed by a more gradual lowering of k_{obs} as the phospholipid is further increased. The steepness of the slopes in the initial stages corresponding to DMPC liposomes is in the order benzocaine > procaine > tetracaine which is also the same order as their partition coefficients whereas the latter segments of the curves are approximately parallel. In contrast, the curve corresponding to benzocaine in DPPC liposomes exhibits a more gradual change indicating that k_{obs}

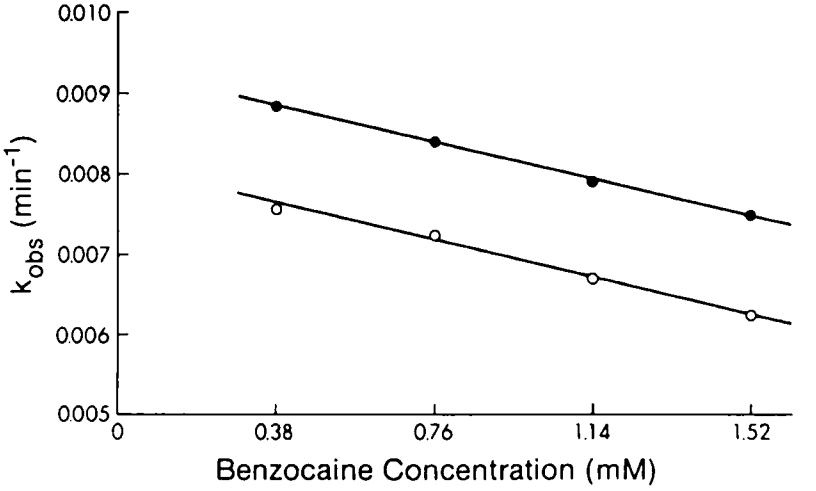


FIGURE 2

Effect of the benzocaine concentration on the hydrolysis rate constant (k_{obs}) in liposomes at pH 12.2 and 30°C. DMPC, O; DPPC, ●.

TABLE 3

Hydrolysis Rate Constants of Benzocaine in DMPC Liposomes (k_{obs}) as a Function of the Ionic Strength of the Medium at pH 12.2 and 30°C.[a]

Ionic Strength	10^3 k_{obs}(SD)
0.046	7.3(0.20)
0.060	7.2(0.19)
0.100	6.9(0.13)
0.154 (isotonic)	6.8(0.09)
0.250	6.8(0.11)

[a]the initial benzocaine concentration was 0.76mM; the DMPC concentration was 2.9mM.

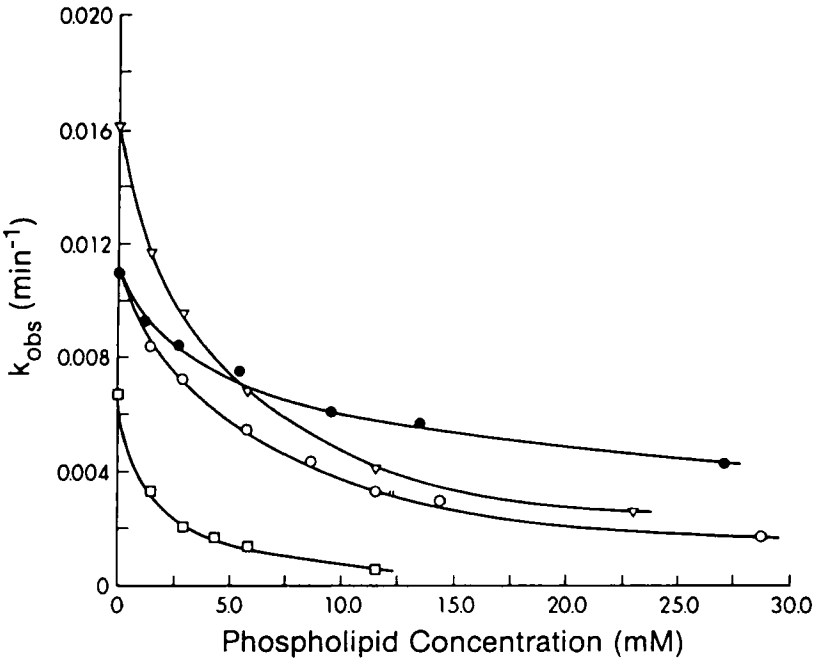


FIGURE 3

Effect of the phospholipid concentration on the hydrolysis rate constant (k_{obs}) of local anesthetics in liposomes at pH 12.2 and 30°C. The initial drug concentration was 0.76mM. Benzocaine (DMPC), ○; (DPPC), ●; procaine(DMPC), ▽; tetracaine (DMPC), □.

is much less influenced by the amount of lipid when the liposomes exist in the gel state. However, it is significant to observe that in fluid DMPC liposomes the relative increase in stability of benzocaine or procaine parallels the increase in f_L (Table 4) whereas approximately a 4-fold increase in the stability of tetracaine is obtained by a 40% increase in f_L at the higher DMPC concentration.

Incorporation of local anesthetics into liposomes for the purpose of stabilization appears also to

TABLE 4

Relationship Between the Initial Fraction in the Lipid Phase(f_L) and the Relative Stability(k_{obs}/k_B) of Local Anesthetics in Liposomes as a Function of the Phospholipid(DMPC) Concentration.[a]

Local Anesthetic[b]	DMPC Conc.(mM)	f_L	k_{obs}/k_B
Benzocaine	2.9	0.28	0.66
	11.5	0.63	0.31
Procaine	2.9	0.26	0.60
	11.5	0.64	0.25
Tetracaine	2.9	0.63	0.31
	11.5	0.88	0.08

[a]experiments were conducted at pH 12.2 and 30°C.
[b]initial concentrations were 0.76mM.

be pH dependent. Thus, the procaine stability data in Table 5 indicates that only undissociated procaine is able to accumulate in the lipid phase when introduced via the aqueous phase because at pH 7.4, at which procaine is 96% ionized (pK_a = 8.8), only 4% increase in stability was obtained. However, most of the increase in the stability of procaine at pH 12.2 is recoverable at pH 7.4 if the drug is incorporated in liposomes via the organic phase during preparation.

DISCUSSION

The kinetics of benzocaine hydrolysis in liposomes of various compositions differed significantly as shown

TABLE 5

Effect of the Method of Incorporation of Procaine in Liposomes on its Stability at 30°C.[a]

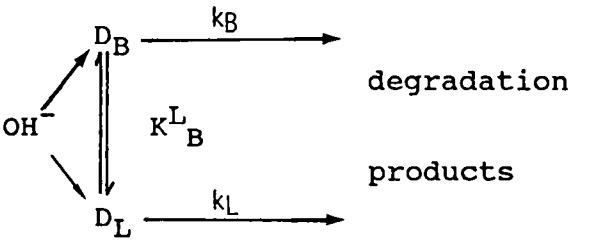
Incorporation of Procaine	% Increase in Stability pH 12.2	pH 7.4
via aqueous phase	41.0	4.0
via organic phase	43.0	30.0

[a]DMPC concentration = 2.9mM; procaine conc. = 0.76mM.

in Table 2. Thus, association of the drug with the lipid phase appears to be responsible for a lower k_{obs} than k_B. A possible reason for the reduced hydrolysis in liposomes is the reduced reactivity in the lipid phase of that fraction of drug associated with it, in particular that which is partitioned deep in the bilayers. Thus, solutes which have a high k_L and a substantial f_L may be predicted to yield the greatest decrease in k_{obs}. Liposomal compositions which augment either of these parameters should provide stability improvement. The influence of f_L on benzocaine stability is well illustrated from the evidence of k_{obs} under the influence of varying concentrations of benzocaine (Fig. 2), DMPC(Fig. 3 and Table 4) and ionic strength (Table 3). However, the respective contributions of k_L and f_L to k_{obs} are not as obvious in liposomes of different compositions.

For example, in liposomes of decreased bilayer fluidity less drug is able to be accommodated but the magnitude of k_L is not necessarily significantly different than in more fluid liposomes. The occurrence of a phase transition from a fluid liquid crystalline state to a more rigid gel-like state is a characteristic of phospholipids and for DMPC[20] the phase transition temperature(T_c) is 23° whereas it is 41° for DPPC[20] and 25°-40° for SPHING[21]. The partition coefficient of solutes in liposomes often exhibits a remarkable decrease upon cooling liposomes below the T_c[22-26] which is believed to be due to the higher energy requirement of accommodation of solute molecules in the rigid gel state of the bilayers[23]. Thus, the increase in stability of benzocaine in DPPC liposomes is lower because less benzocaine associates with these bilayers. In a similar fashion reduced benzocaine accommodation in DMPC:CHOL liposomes, due to the decreased fluidity[1], contributes to its lower observed stability. Reduced binding, partitioning and stability of solutes in fluid liposomal membranes to which CHOL has been added has been previously reported[3,25-28]. Although there is no direct evidence of the reactivity in PS or SPHING liposomes, differences in permeability to OH^- may be a major contributing factor. In the case of PS liposomes, strong negatively-charged sites at the liposome surfaces repel OH^- and, therefore, catalytic attack of the reactive centres of benzocaine molecules partitioned in the bilayers is reduced. In contrast, the permeability of SPHING bilayers, like other phospholipids, is greater in the region of its T_c[19] with the result of increased OH^- penetration and hydrolytic attack of benzocaine molecules in the bilayers.

The degradation of the local anesthetics in liposomes may be described by a simple kinetic model as depicted in Scheme 1:



Scheme 1

where D_B and D_L correspond to drug in the aqueous buffer solution and the lipid phase, respectively. The overall rate of hydrolysis is expressed accordingly[14]:

$$-(V_B+V_C)\frac{dC}{dt} = k_B V_B C_B + k_L V_L C_L \qquad (7)$$

where V_B and V_L are the volumes of the aqueous and lipid phases, respectively, C_B and C_L are the concentrations of drug in each phase, and C refers to the total concentration in the system. V_L was estimated from the density of 1.02 of hydrated lipid[3]. Since $K^L_B = C_L/C_B$, then Eq.(7) may be expressed as

$$\frac{-d\ln C}{dt} = \frac{k_B - k_L}{1+K^L_B \frac{V_L}{V_B}} + k_L = k_{obs} \qquad (8)$$

and following re-arrangement gives

$$k_{obs} + \frac{k_{obs}}{K^{L}_{B}\frac{V_L}{V_B}} = \frac{k_B}{K^{L}_{B}\frac{V_L}{V_B}} + k_L \qquad (9)$$

A plot of this linear relationship yields k_B and k_L from the slope and intercept, respectively, and for benzocaine, procaine, and tetracaine these are shown in Fig. 4. Table 6 compares k_B determined from Eq.(9) with k_B obtained experimentally. The excellent agreement found supports the validity of Scheme 1 in describing the events occurring during the degradation of these three local anesthetics. Values of k_L determined from the intercepts were very low (in the order of 10^{-4} - 10^{-5} min^{-1}) and were only about 0.5 - 1.0% of k_B indicating a strong protective capability of liposomes against hydrolysis of local anesthetics. A comparison of the reactivity in the lipid phase to that in the total liposomal suspension (last column, Table 6) not only emphasizes the impact of a low k_L but also the significance of a higher f_L of tetracaine with respect to the observed increase in its stability.

The stabilization of undissociated drugs such as the local anesthetics under the present conditions would appear to be strongly influenced by K^{L}_{B}. For example, values of K^{L}_{B} for benzocaine, procaine, and tetracaine of 169, 180, and 873 correspond to a 34%, 41%, and 69% increase in stability, respectively. A test of such a correlation is demonstrated in Fig.5 which also includes data points for other local anesthetics and solutes whose stabilities in liposomes

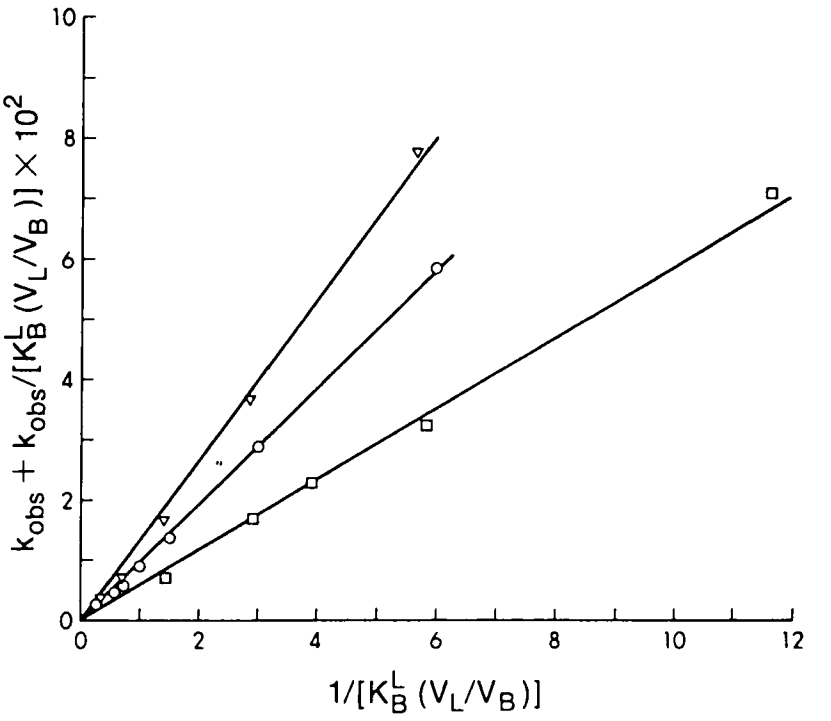


FIGURE 4

Plots of Eq.(9) for benzocaine, O; procaine, ∇; tetracaine, □. A multiplication factor of 10 was applied to both axes for tetracaine.

TABLE 6

Comparisons of Hydrolysis in Aqueous Buffer Solution (k_B), Hydrolysis of Associated Drug Relative to Free (k_L/k_B) and Contribution of the Phospholipid Phase to Hydrolysis in the Liposomal Suspension ($k_L f_L/k_{obs}$)

Local Anesthetic	$k_B(min^{-1})$ (calc'd)	$k_B(min^{-1})$ (expt'l)	10^2 k_L/k_B	10^3 $k_L f_L/k_{obs}$
Benzocaine	0.010	0.011	1.2	4.5
Procaine	0.014	0.016	1.0	4.3
Tetracaine	0.006	0.007	0.4	8.4

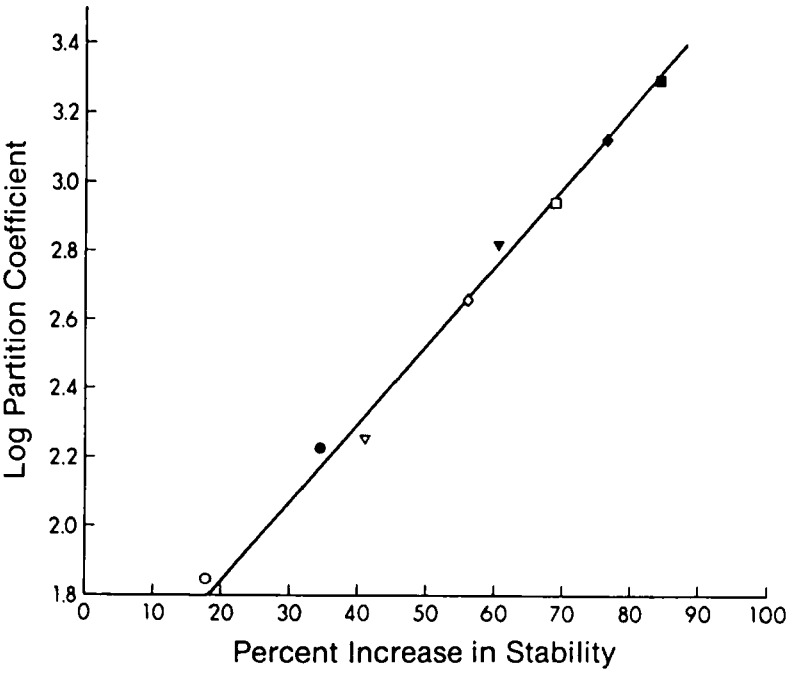


FIGURE 5

Correlation of the partition coefficient (K^{L}_{B}) and the observed percent increase in stability of various local anesthetics and other solutes in DMPC liposomes at pH 12.2 and 30°C. The curve was determined using linear regression analysis (r=0.996). Methyl p-amino-benzoate, ○; benzocaine, ●; procaine, ▽; n-propyl p-amino benzoate, ▼; tetracaine, □; n-butyl p-amino-benzoate, ■; 2-diethylaminoethyl p-nitrobenzoate (ref. 4), ◇; indomethacin(ref. 5), ◆.

have been previously reported[4,5]. An excellent correlation (r = 0.996) was obtained suggesting the possibility of predicting the degree of stabilization of a drug associated with a liposome phase from its liposome K^{L}_{B}.

Similarities between micellar and liposome systems have been referred to previously[5]. However, phospho-

TABLE 7

Comparison of the Micellar and Liposomal Stabilization of Local Anesthetics

Local Anesthetic	% Increase in Stability		
	Surfactant[a]	Micelles (ref.)	Liposomes[b]
Benzocaine	POE 24 monocetyl ether (~4.5mM)	39.4(14)	34.0
	C-30 (~12mM)	58.0(16)	34.0
	SLS (9.3mM)	35.6(16)	34.0
Procaine	PLE (8.3mM)	50.5(15)	41.0
	SLS (10mM)	65.3(15)	41.0
	CTAB (4mM)	39.1(15)	41.0
	N-dodecyl betaine (2mM)	18.3(15)	41.0
n-Butyl-p-amino benzoate	POE 24 monocetyl ether (~4.5mM)	55.7(14)	84.5

[a]C-30 = Cetyl alcohol polyoxyethylene ethers
SLS = Sodium lauryl sulfate
PLE = Polyoxyethylene lauryl ether
CTAB = Cetyltrimethylammonium bromide
[b]DMPC concentration = 0.2% (= 2.9mM)

lipid bilayers also have properties characteristic of lyotropic smectic mesophases[17,30]. An indication of which physical state is most predominant in stabilizing drugs is given by a comparison of the stabilization of local anesthetics in liposomes and surfactant micelles given in Table 7. On a mole per mole basis stabilization in liposomes on average is approximately 50% greater than in micellar systems and this would suggest that the liposomal bilayers in the liquid crystalline state may have some characteristics of a lyotropic smectic mesophase. For example, the hydrolysis reaction rate of procaine hydrochloride in lyotropic liquid crystalline phases of polyoxethylene tridecyl ether was found to be approximately 300-fold slower than in aqueous media[17].

It appears that ionized drugs may also be stabilized in liposomes but only if they have hydrophobic moieties in their structures large enough to outweigh the surface polar group interactions and partition in the hydrocarbon regions of the bilayers, such as occurs with indomethacin[5]. Otherwise, drugs such as procaine[3] or ASA[10] are stabilized only if they are first incorporated directly into the lipid phase prior to liposome formation(Table 5). In this case, the decreased reactivity in the lipid phase is probably due to a more favorable orientation of the drug molecules deeper in the bilayers which are anchored by electrostatic association of the polar groups of the drug with those of the phospholipid molecules in the surface regions of the liposomes.

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