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Kinetics of hydrolysis and stabilization of acetylsalicylic acid in liposome formulations

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Summary

The stability of acetylsalicylic acid (ASA) has been determined in liposome systems under various conditions of liposome composition, pH, ionic strength, temperature, liposome and ASA concentrations. ASA was preferentially stabilized in its anionic form, although a larger fraction of ASA was associated with the lipid phase (f_L) of the liposomes at low pH. Liposomes possessing increased bilayer rigidity resulted in a lower f_L and an increased pseudo-first-order hydrolysis rate constant (k_{obs}) of ASA. Increasing the ionic strength decreased R_k ($= k_{obs}/k_B$, where k_B is the rate constant in aqueous buffer solution) only under hypertonic conditions. R_k was lowest in the vicinity of the phase transition temperature (T_c) of the phospholipid. Significant stabilization of ASA was observed only if the drug was incorporated into liposomes via the organic phase. The addition of stearylamine to liposomes resulted in a substantially greater degree of stabilization of anionic ASA whereas adding an excess of salicylic acid increased k_{obs} to values near k_B. It is concluded that the binding of anionic ASA to positive centers of charge located below the surfaces of the bilayers protects ASA from base-catalyzed hydrolysis. The degree of stabilization of ASA is, in most cases, proportional to f_L. Thus, methods used to increase f_L or maintain a fairly constant f_L for longer periods of time should result in useful stabilized preparations of ASA.

Introduction

Among the several potential applications of liposomes in formulation practice is the possibility of stabilizing drugs in aqueous solution. Liposomes are colloidal dispersions of phospholipids which have the capacity to permit partitioning, binding, or entrapment of solutes in a manner analogous to that in micellar systems. Thus, the solute may become protected against environmental influences such as hydrolytic degradation. However, liposomes are organized differently than micelles and may confer different degrees of stability to solutes on a mol per mol basis.

Only a few studies on the stabilization of esters or amides in liposomes have been reported. The stability of procaine was increased in egg lecithin liposomes (Yotsuyanagi et al., 1979a) whereas 2-diethylaminoethyl *p*-nitrobenzoate underwent either hydrolysis rate retardation or enhancement depending on the pH of the system (Yotsuyanagi et al., 1979b). Liposomes of egg lecithin were also interacted with 3 trimethylammonium halide es-

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ters and varying degrees of stability were found (Fatah and Lowe, 1983). The hydrolysis rate of indomethacin at pH 9.37 was reduced by 80% in neutral, negatively-charged and positively-charged egg lecithin liposomes, the stability of cyclocytidine was unaffected and positively-charged liposomes increased the rate of hydrolysis of *p*-nitrophenyl acetate (D'Silva and Notari, 1982). However, the stability of *p*-nitrophenyl acetate was increased in neutral or negatively charged liposomes. The hydrolysis rate constant of this ester increased as a function of the egg lecithin concentration in unilamellar liposomes suggesting a surface reaction was responsible for its catalytic degradation (Yotsuyanagi and Ikeda, 1980).

The stability of acetylsalicylic acid (ASA) in surfactant systems has been reported previously (Nogami et al., 1962; Mitchell and Broadhead, 1967). This report is concerned with the determination of the extent of stabilization of ASA in liposomes under various conditions of preparation, pH, liposome composition and concentration, ASA concentration, ionic strength, and temperature.

Materials and Methods

Materials

ASA (99%) was obtained from Aldrich Chemical Co.; L-α-Dimyristoylphosphatidylcholine (DMPC, 98%), L-α-dipalmitoylphosphatidylcholine (DPPC, 99%), L-α-phosphatidylcholine (EPC) type V-E from egg yolk, stearylamine (STEAR), dicetylphosphate (DCP), cholesterol (CHOL), and sphingomyelin (SPHING) were purchased from Sigma Chemical Co. All other chemicals and solvents were reagent grade. Water was glass-distilled.

Methods

Aqueous buffer solutions were prepared as follows: pH 1.0 (0.108 M HCl + 0.05 M KCl); pH 2.4 (0.094 M citric acid + 0.012 M Na_2HPO_4); pH 4.0 (0.16 M acetic acid + 0.036 M sodium acetate); pH 6.0 (0.059 M KH_2PO_4 + 0.007 M Na_2HPO_4); pH 8.0 (0.0025 M KH_2PO_4 + 0.064 M Na_2PO_4); pH 9.4 (0.100 M boric acid + 0.101 M sodium carbonate). The ionic strength of each buffer was adjusted to 0.15 by the addition of NaCl unless otherwise specified.

Preparation of liposomes. Thin films of lipids were formed on the inside of 1 litre round-bottom flasks by adding the lipids to the flasks, dissolving them in chloroform, removing the solvent by rotary evaporation at 40 °C, flushing with N_2 gas then drying overnight under vacuum at 40 °C. Subsequently, liposomes were formed by adding aqueous buffer solution at a temperature at least 10 °C above the phase transition temperature (T_c) of the phospholipid. The contents were vortex-mixed for 10 min or until all of the lipid had been removed from the walls of the flask and a homogeneous dispersion of liposomes had formed. The concentrations of lipid and ASA were 14.4 mM and 7.5 mM, respectively, unless otherwise specified. Normally, ASA was initially incorporated in the film with the lipids, but some experiments were conducted by including the ASA in the aqueous buffer solution during formation of the liposomes.

Kinetic studies and analysis. The stock liposome preparation was divided into 2 ml portions, placed in capped vials, and maintained at 30 °C in a water-bath. At various time intervals, samples were quantitatively transferred to 25 ml volumetric flasks and diluted to volume with isopropyl alcohol (which yielded a clear solution) and then assayed spectrophotometrically (Pye Unicam, SP6-550 spectrophotometer) for salicylic acid (SA) at $\lambda = 303$ nm. No interference in the analysis of SA was found by the presence of ASA, lipids or buffering agents in the solution. The degradation of ASA in aqueous buffer solution was always followed simultaneously as a control.

Since 1 mol of ASA yields 1 mol of SA in the hydrolysis reaction, the concentration of ASA remaining in a sample at any given time, $[ASA]_t$, was calculated from

$$A_\infty = \epsilon_{SA} \cdot [ASA]_0 \quad (1)$$

and

$$(A_\infty - A_t)\alpha[ASA]_t \quad (2)$$

where A_∞ is the theoretical absorbance of SA at time ∞, $[ASA]_0$ is the initial ASA concentration, ϵ_{SA} is the molar absorptivity of SA, and A_t is the absorbance of SA at any given time (Nogami et al., 1962; Parrott, 1976; Mitchell and Broadhead, 1967; Blanch and Finch, 1971).

The kinetics of hydrolysis of ASA associated with the lipid phase only was determined experimentally by first preparing liposomes containing 28.8 mM of lipid and 15 mM ASA, centrifuging (135,000 × *g*, 30 °C; Beckman Model L8-55 Ultracentrifuge), removing the supernatant, resuspending with aqueous buffer solution and again centrifuging and resuspending for a total of 3 washings, and finally diluting to 14.4 mM phospholipid. In these experiments, $[ASA]_0$ must be determined by analysis. However, the absorbance of SA interferes with the absorbance of ASA but a procedure involving simultaneous equations may be used (Pernarowski, 1969; D'Silva and Notari, 1982). Once $[ASA]_0$ was determined the degradation of ASA in the lipid phase was followed in the usual manner.

The fraction of ASA associated with the lipid phase (f_L) was measured by analyzing the supernatant and the pellet of each liposome sample after a single centrifugation (135,000 × *g*) and separation of the phases by careful decantation of the supernatant.

Results and Discussion

The hydrolysis of ASA in control buffer solutions or liposomes was followed for at least 3 half-lives and found to obey pseudo-first-order kinetics ($r > 0.990$). Thus, rate constants were obtained from

$$\log[ASA]_t = \log[ASA]_0 - \frac{kt}{2.3} \qquad (3)$$

In control buffer solutions, the pseudo-first-order hydrolysis rate constant, $k = k_B$ and in liposomes $k = k_{obs}$. A comparison of k_B and k_{obs} in DMPC liposomes at different pH values is shown in Fig. 1. The pH–rate profiles for ASA are similar to

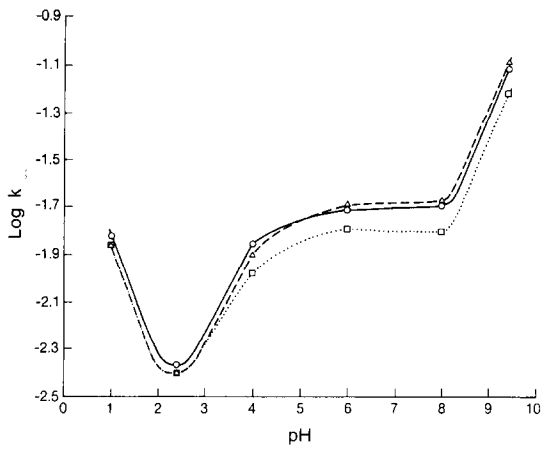

Fig. 1. pH–rate profiles of the hydrolysis of ASA at 30 °C in aqueous buffer solutions (○), in DMPC liposomes in which ASA was incorporated via the aqueous phase (△), and in DMPC liposomes in which ASA was incorporated via the organic phase (□).

that which had been previously established by others (Edwards, 1950). Over the range pH 1–3, k_{obs} was slightly lower than k_B (Student's *t*-test, $P < 0.10$) but at higher pHs k_{obs} was dependent on the manner in which ASA was incorporated into the liposomes. When ASA was added initially via the organic phase, the stability of ASA was enhanced but when ASA was incorporated via the aqueous phase, ASA was not stabilized. These differences were not observed at pH < 3. It should be noted that when ASA was incorporated in liposomes via the organic phase, k_{obs} was consistently reduced at pH 4.0 and at higher pHs by 22–25% compared to the control buffer solution ($P < 0.05$). In contrast, when ASA was introduced via the aqueous phase at pH 8.0, k_{obs} was greater than k_B probably due to adsorption and orientation of ASA at the liposome surfaces which increased the susceptibility of ASA to OH^- attack. A similar behavior has been observed for 2-diethylaminoethyl *p*-nitrobenzoate (Yotsuyanagi et al., 1979b).

At equilibrium following liposome formation the total amount of ASA in the liposomes, $(ASA)_T$, is equal to the sum of drug in the lipid phase, $(ASA)_L$ and the aqueous buffer phase $(ASA)_B$,

TABLE 1

Comparison of the first order hydrolysis rate constant of ASA in DMPC liposomes (k_{obs}), lipid phase (k_L), aqueous buffer (k_B) and of the fraction of ASA associated with the lipid phase (f_L)

pH	k_{obs} (h^{-1})	k_L (h^{-1})	k_B (h^{-1})	f_L
1.0	$0.0139(4\times10^{-5})$	$0.0112(3\times10^{-4})$	$0.0151(4\times10^{-4})$	0.325(0.009)
4.0	$0.0105(5\times10^{-4})$	$0.0030(8\times10^{-5})$	$0.0139(2\times10^{-4})$	0.258(0.013)
8.0	$0.0157(2\times10^{-4})$	$0.0011(7\times10^{-5})$	$0.0204(9\times10^{-4})$	0.196(0.004)

[DMPC] = 14.4mM; $[ASA]_0$ = 7.5mM; 30 °C. Standard deviations shown in brackets; $n = 3$.

respectively. Hence, the loss of total ASA in liposomes is given by

$$-\frac{d(ASA)_T}{dt} = k_{obs}(ASA)_T$$

$$= k_L(ASA)_L + k_B(ASA)_B \quad (4)$$

and,

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

where $(ASA)_L/(ASA)_T = f_L$ and $(ASA)_B/(ASA)_T = f_B \cdot k_L$ is the pseudo-first-order rate constant of hydrolysis of ASA in the lipid phase. Substituting $f_B = 1 - f_L$ and rearranging Eqn. 5 yields

$$k_{obs} = k_B + f_L(k_L - k_B) \quad (6)$$

Thus, the greater the difference between k_L and k_B and the larger is f_L, the greater the stabilization of drug by liposomes. Table 1 compares the magnitudes of each of these parameters at 3 pH values. As the pH increases, k_L decreases but f_L also decreases. This is indicative of a change from a hydrophobic partitioning mechanism of incorporation of unionized ASA (ASAH) to an electrostatic binding mechanism of anionic ASA (ASA^-) to phospholipid resulting in considerably reduced levels of incorporation. At the same time, bound ASA is stabilized against catalytic hydrolysis. The relatively low stabilization of ASA in liposomes at pH 1.0 can be explained by a low association constant with liposomes resulting in rapid diffusion of ASA from the lipid phase to the external aqueous buffer solution as degradation proceeds.

The effects of varying the concentration of ASA or DMPC on the relative stabilities of ASA are shown in Table 2 and Fig. 2, respectively. As the $[ASA]_T$ increases the changes of k_{obs} and k_B appear to follow different trends but comparisons of $k_{obs}/k_B = R_k$ in Table 2 suggest a decreasing

TABLE 2

Hydrolysis of ASA in DMPC liposomes as a function of ASA concentration of pH 4.0 and 30 °C

ASA conc. (mM)	k_{obs} (h^{-1})	k_B (h^{-1})	$R_k (= k_{obs}/k_B)$
3.75	$0.0101(2\times10^{-4})$	$0.0134(2\times10^{-4})$	0.754
7.50	$0.0105(5\times10^{-4})$	$0.0139(2\times10^{-4})$	0.755
15.0	$0.0120(3\times10^{-4})$	$0.0128(3\times10^{-4})$	0.781
30.0	$0.0092(2\times10^{-4})$	$0.0111(4\times10^{-4})$	0.829

[DMPC] = 14.4 mM. Standard deviations shown in brackets; $n = 3$.

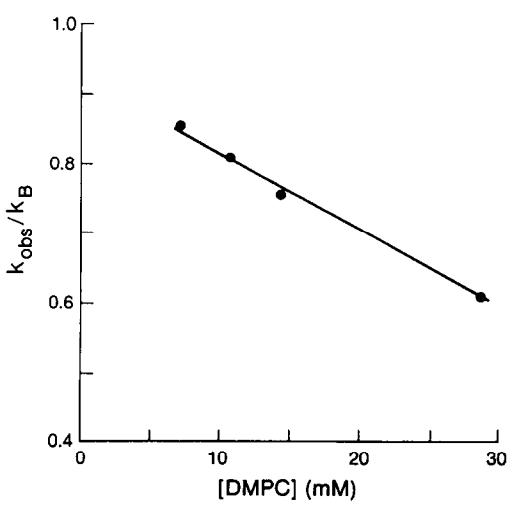

Fig. 2. The effect of DMPC concentration on the relative increase in stability against hydrolysis of ASA in DMPC liposomes at pH 4.0 and 30 °C. The initial concentration of ASA was 7.5 mM.

TABLE 3

Hydrolysis of ASA in DMPC liposomes as a function of the ionic strength of the medium of pH 4.0 and 30°C

Ionic strength [b]	k_{obs} (h^{-1})	k_B (h^{-1})	$R_k (= k_{obs}/k_b)$
0.07	$0.0106(5\times10^{-4})$	$0.0140(2\times10^{-4})$	0.757
0.15	$0.0105(5\times10^{-4})$	$0.0139(1\times10^{-4})$	0.755
0.35	$0.0099(8\times10^{-5})$	$0.0141(5\times10^{-5})$	0.702
0.63	$0.0099(5\times10^{-5})$	$0.0143(2\times10^{-4})$	0.692

Standard deviations shown in brackets; $n = 3$.
[a] [DMPC] = 14.4 mM; initial [ASA] = 7.5 mM.
[b] Adjusted with NaCl.

role of liposomes to stabilize ASA. On the other hand, increasing the [DMPC] decreases R_k linearly as shown in Fig. 2. It was also determined that k_L was essentially the same at 14.4 mM and 28.8 mM DMPC. These data are consistent with the argument that the extent of stabilization of ASA in liposomes is determined by the number of binding sites available for ASA^-. The observation that extrapolation of the curve to zero [DMPC] does not intersect at $R_k = 1.0$ suggests that a threshold [DMPC] is required before binding of ASA^- becomes proportional to the [DMPC], and partly because some of the ASA which is unionized is undergoing partitioning.

In comparison to pH, environmental changes in the liposomes have a smaller influence on the stabilization of ASA as shown in Tables 3 and 4. Effect of ionic strength on R_k is seen to occur only in hypertonic media and the trend is towards decreasing R_k values. Kano and Fendler (1979) have shown that there is a substantial increase in microviscosities on osmotic shrinkage of liposomes and at an ionic strength equivalent to 1 M NaCl there is no free water in the vesicles. Therefore, the properties of the liposomes under these conditions should lead to reduced hydrolysis of ASA which is bound to phospholipid below the surfaces of the bilayers. Similarly, differences in hydration and packing of the molecules above and below the T_c may influence the hydrolysis of ASA. For instance, in the gel state below the T_c steric hindrances and structured water throughout the bilayers would have a tendency to decrease f_L and, hence, k_{obs} whereas above the T_c water is more strongly bound to the fluid-state bilayers (Enders and Nimtz, 1984) thus increasing the exposure of liposome-associated ASA to catalytic hydrolysis. The results given in Table 4 show the highest values of R_k at the lowest and highest temperatures studied. Increasing the temperature through the T_c gradually increases the stabilization of ASA until above 30°C a fairly abrupt change in the stabilization process takes place as the thermal kinetic energies of the molecules and hydrational influences continue to increase. Between 20 and 30°C minimal conditions of steric hindrance and hydration may be contributing to maximum stabilization of ASA in DMPC liposomes.

TABLE 4

Hydrolysis of ASA in DMPC liposomes as a function of temperature at pH 4.0

Temp. (°C)	k_{obs} (h^{-1})	k_B (h^{-1})	$R_k (= k_{obs}/k_B)$
10	$0.0012(9\times10^{-5})$	$0.0015(1\times10^{-4})$	0.800
20	$0.0037(9\times10^{-5})$	$0.0046(1\times10^{-4})$	0.789
30	$0.0105(5\times10^{-4})$	$0.0139(1\times10^{-4})$	0.755
40	$0.0286(2\times10^{-4})$	$0.0351(8\times10^{-4})$	0.814
50	$0.0674(4\times10^{-4})$	$0.0817(5\times10^{-4})$	0.825

[DMPC] = 14.4 mM; initial [ASA] = 7.5 mM. Standard deviations shown in brackets; $n = 3$.

Table 5 shows the effects of various liposome compositions on the stabilization of ASA. Small differences in R_k are observed by introducing hydrocarbon chain unsaturations, such as occur with EPC and SPHING, or increased packing of the molecules in the bilayers and a negative surface charge as occurs upon the addition of DCP. On the other hand, liposomes of DPPC are in a gel state at 30°C and liposomes containing CHOL are less fluid, more structured, and more highly hydrated (Papahadjopoulos and Kimelberg, 1973; Ter-Minassian-Saraga and Madelmont, 1982). As discussed earlier, such conditions cause f_L to decrease (as shown in Table 5 for DMPC : CHOL (3 : 1) liposomes) and a significant increase in R_k is observed compared to the other compositions.

The effect of some specific compositional changes to DMPC liposomes on f_L and the contribution of the lipid phase to k_{obs} are described

TABLE 5

Hydrolysis of ASA in liposomes of various compositions at pH 4.0 and 30 °C

Liposome composition	k_{obs} (h^{-1})	$R_k (= k_{obs}/k_B)$
Group A		
DMPC	$0.0105(5\times10^{-4})$	0.755
EPC	$0.0111(6\times10^{-4})$	0.799
SPHING	$0.0112(2\times10^{-4})$	0.806
DMPC:DCP(2:1)	$0.0112(1\times10^{-4})$	0.806
DMPC:EPC(1:1)	$0.0108(2\times10^{-4})$	0.777
Group B		
DPPC	$0.0116(2\times10^{-5})$	0.835
DMPC:CHOL(3:1)	$0.1210(8\times10^{-5})$	0.871

Total lipid concentration = 14.4 mM; mol ratios shown in brackets. Standard deviations shown in brackets beside k_{obs} values; ($n = 3$). Group A and Group B are significantly different at $P < 0.01$

in Table 6. An apparent correlation exists providing strong evidence that the protection of ASA by the lipid phase against hydrolysis as reflected in k_L remains essentially constant and that the effect of the additives is mainly to alter the number of available binding sites for ASA^-. Furthermore, the close agreement between k_{obs}(expt.) and k_{obs}(calc'd. from Eqn. 6) illustrates that k_L as determined in DMPC liposomes may be used to adequately predict k_{obs} in other liposome compositions. It can also be seen that the addition of STEAR increases f_L and, therefore, the hydrolysis reactivity in the lipid phase presumably due to the presence of additional binding sites available to ASA^-. In contrast, the addition of excess SA

TABLE 6

Contribution of the lipid phase to the hydrolysis reactivity of ASA in liposome formulations at pH 4.0 and 30 °C

Liposome formulation [a]	k_{obs}(calc'd) (h^{-1})	k_{obs}(expt.) (h^{-1})	f_L	$k_L f_L / k_{obs}$
DMPC	0.0111	0.0105	0.258	0.074
DMPC:CHOL(3:1)	0.0126	0.0121	0.117	0.029
DMPC + 2.7 mM salicylic acid	0.0122	0.0126	0.153	0.036
DMPC:STEAR(2:1)	0.0101	0.0080	0.349	0.131

[a] Total lipid conc. = 14.4 mM (mol ratios shown in brackets); $[ASA]_0 = 7.5$ mM.

reduced f_L and the liposome stabilization of ASA. It may be concluded that the SA competes with ASA for the available binding sites on the phospholipid molecules. Thus, this may also be a factor in the stabilization of ASA at later times in DMPC liposomes when a significant amount of ASA has degraded to SA.

The stabilization of drugs associated with liposomes under various conditions can depend on the integrity and stability of the liposome itself. Any changes which may occur in the architecture of the liposome can influence the extent of association of solute and, therefore, its rate of degradation through the f_L term. The liposomes in these studies did not undergo any obvious visual changes when subjected to variations in pH, temperature, or ionic strength and at pHs at which ASA was ionized its stability was little affected by such variations in the conditions of the system. Even though there were possible changes in the particle size distribution during the reaction time, this, in itself, had no effect on the kinetics at pH 4.0 or higher since f_L was found to be mainly dependent on an electrostatic binding mechanism.

A comparison of the stabilization of ASA in liposomes and micelles shows certain similarities. For example, the hydrolysis of unionized ASA was suppressed by anionic, cationic, and non-ionic surfactants in micellar solutions but the anionic form was suppressed only by micelles of cationic surfactants (Nogami et al., 1962). However, on a mol per mol basis a 16:1 ratio of sodium lauryl sulfate:ASA is required to provide a level of stability of ASA and pH 4.0 equivalent to that of a 4:1 ratio of DMPC:ASA. In another example DMPC liposomes are approximately 2.5 times more efficient than cetomacrogol micelles in stabilizing ASA at pH 4.0 but at pH 1.0 the efficiencies of stabilization of ASA in cetomacrogol micelles and liposomes appear to be approximately similar (Mitchell and Broadhead, 1967). The possibility of further improving the stability of ASA in liposomes increases if changes in composition which offer additional binding sites are made, better methods of liposome preparation are found, or liposomes are coated with a substance which retards diffusion in and out of the bilayers, such as may be provided by a polymer.

This latter approach is currently under investigation.

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