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Hydrolysis and Stability of Acetylsalicylic Acid in Stearylamine-containing Liposomes

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Abstract—The hydrolysis and the stabilization of acetylsalicylic acid (ASA) in liposomes at 30°C were studied. The liposomes consisted of dimyristoylphosphatidylcholine (DMPC) and stearylamine. At pH 4·0 and 8·0, the pseudo-first-order rate constants (k_{obs}) in DMPC: stearylamine (2:1 mole ratio) liposomes were approximately 60% of the values in control solutions (k_B) if ASA was incorporated via the organic phase. In contrast, when ASA was added via the aqueous phase, $k_{obs} = k_B$ at pH 4·0 but $k_{obs} < k_B$ at pH 8·0 and k_{obs} increased with the fraction of stearylamine in the liposomes. However, when ASA was added via the organic phase, reactions occurred which resulted in the loss of ASA as a function of the time period between phase admixture and the point of film hydration. A product of the reactions was determined by IR and TLC to be *N*-stearylacetamide. Both the initial loss of ASA and the increase in stability decreased as the DMPC: stearylamine mole ratio increased. A mechanism of aminolysis occurring in the organic solvent and at liposome surfaces between ASA and stearylamine or DMPC at pH 8·0 has been suggested. It is concluded that the orientation of ASA and the ordered structural environment of the bilayers minimizes the aminolytic and hydrolytic reactions.

The application of liposomes as potential drug delivery systems has been widely studied. However, the possibility of stabilizing drugs or enzymes with liposomes has been sparsely investigated (Habib & Rogers 1988; Yotsuyanagi et al 1979a, b; Kurosaki et al 1981; D'Silva & Notari 1982; Pourkavoos et al (1984). The stabilization of drugs in liposomes appears to be highly dependent on the degree of association of the drug with the liposome bilayers and is a function of the hydrophobicity of the drug, pH for ionizable drugs, and the composition of the liposomes (Habib & Rogers 1988; Yotsuyanagi et al 1979a; Kurosaki et al 1981; D'Silva & Notari 1982). Furthermore, it appears that the deeper the reactive centre of an ester or amide in the bilayer, the less susceptible it is to hydrolytic attack (Fatah & Loew 1983). In contrast, drug molecules which are adsorbed at the surfaces may exhibit an increased degradative rate in alkaline media (Yotsuyanagi & Ikeda 1980; Kurosaki et al 1981). In our previous study, it was found that although the stability of acetylsalicylic acid (ASA) in liposomes of various compositions differed only slightly, it was higher than in aqueous solutions (Habib & Rogers 1988). However, inclusion of stearylamine in dimyristoylphosphatidylcholine (DMPC) liposomes yielded varying results of ASA stability. This study was therefore undertaken to examine the potential of DMPC: stearylamine liposomes to stabilize ASA and to determine their mechanism of degradation.

Materials and Methods

Materials

Acetylsalicylic acid (ASA, 99+%, Gold Label) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Salicylic acid and *p*-chloroaniline were from J. T. Baker Chemical Co. (Phillipsburg, NJ), L-α-Dimyristoylphosphatidylcholine (DMPC, 98%), cholesterol (99+%), and stearylamine (90%) were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals and solvents were reagent grade. Water was deionized and glass-distilled.

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Liposome preparation

Liposomes were prepared as previously described (Habib & Rogers 1988). The initial concentrations of lipid and ASA were 14·4 and 7·5 mM, respectively, in all liposome experiments. For determination of ASA in the lipid phase, the concentrations were doubled initially before dilution. Normally, ASA was combined with the lipids in the chloroform phase; however in some experiments, ASA was added in the aqueous buffer solution (0·16 M acetic acid + 0·036 M sodium acetate, pH 4·0, or 0·0025 M KH_2PO_4 + 0·064 M Na_2HPO_4, pH 8·0) adjusted to 0·15 M ionic strength with NaCl. When employed in experiments, salicylic acid was added in the organic phase of lipids.

Synthesis and characterization of N-*stearylacetamide*

Molar equivalents of stearylamine and acetic anhydride or ASA were allowed to react overnight in chloroform solution at room temperature (21°C) with continuous shaking. This was then washed with 3 × 15 mL of cold 10% HCl in a separatory funnel to remove excess organic base as HCl salts. The chloroform layer was further dried with anhydrous sodium sulphate then subjected to rotary evaporation. The dry residue obtained (*N*-stearylacetamide) was recrystallized three times from methanol then stored in powdered form. Qualitative thin-layer chromatography tests for *N*-stearylacetamide were performed on silica gel TLC plates (10 × 10 cm × 2 mm; 60 F254, E. Merck, Germany) using chloroform: methanol (9:1, v/v). Approximately 200 μg of stearylamine or *N*-stearylacetamide, synthesized from acetic anhydride or ASA in chloroform, was spotted and the plates were developed for 5 min. Detection of spots was by UV or iodine vapour.

Samples were also analysed by IR spectroscopy using the pressed disc method (Coutts 1969).

Kinetic studies and analysis

A spectrophotometric analysis of salicylic acid at 303 nM in isopropanol solution has been previously described (Habib & Rogers 1988). Since one mole of ASA yields one mole of salicylic acid in the hydrolytic reaction, the concentration of ASA remaining in a sample at any given time, $[ASA]_t$, was calculated from:

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

and

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

where A_∞ is the theoretical absorbance of salicylic acid at infinite time, $[ASA]_0$ is the initial ASA concentration, ε_{SA} is the molar absorptivity of salicylic acid, and A_t is the absorbance of salicylic acid at any given time. Pseudo-first-order plots yielded values of k_{obs}, the degradation rate constant of ASA in liposomes, and comparisons of the effects of DMPC:stearylamine liposome compositions on the stabilization of ASA were made based on calculations according to equation 3:

$$\% \text{ increase in stability} = \frac{k_B - k_{obs}}{k_B} \times 100 \quad (3)$$

where k_B is the pseudo-first-order rate constant of ASA in aqueous solution. All kinetic studies were conducted only at 30°C since Habib & Rogers (1988) reported that temperature variations did not influence the stabilization of ASA.

Results and Discussion

The kinetics of hydrolysis and stabilization of ASA in liposomes as previously reported (Habib & Rogers 1988) was found to be influenced by the method of preparation of liposomes, liposome composition, and the pH. In particular, it was observed that when ASA was initially added in the aqueous phase (method I), $k_{obs} = k_B$ at pH 4·0 but $k_{obs} < k_B$ at pH 8·0. On the other hand, when ASA was initially added in the organic phase (method II), $k_{obs} < k_B$ and approximately 25% increase in stability of ASA was obtained at either pH 4·0 or 8·0. However, when a positively-charged surface of liposome was prepared by adding stearylamine to DMPC, it was found that the interaction of ASA with this lipid composition led to an initial increase in hydrolysis, followed by increased stabilization as compared with the control ASA solution. The results are illustrated in Figs 1, 2 and in Table 1.

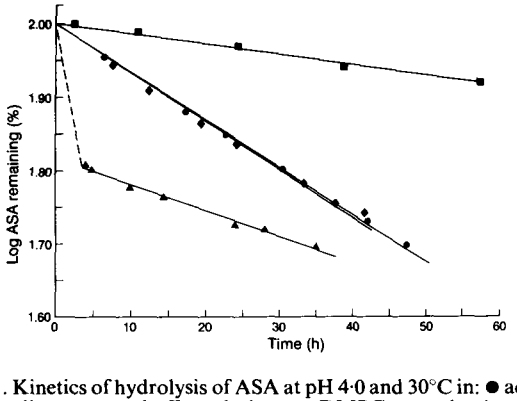


FIG. 1. Kinetics of hydrolysis of ASA at pH 4·0 and 30°C in: ● acetic acid–sodium acetate buffer solution; ◆ DMPC:stearylamine (2:1 mol ratio) liposomes in which ASA was incorporated in the aqueous phase; ▲ DMPC:stearylamine (2:1 mol ratio) liposomes in which ASA was incorporated in the organic phase; ■ DMPC:stearylamine (2:1 mol ratio) liposomes in which the ASA was incorporated in the organic phase, the unentrapped ASA was removed in the supernatant after centrifugation followed by re-suspension of the pellet in the aqueous solution.

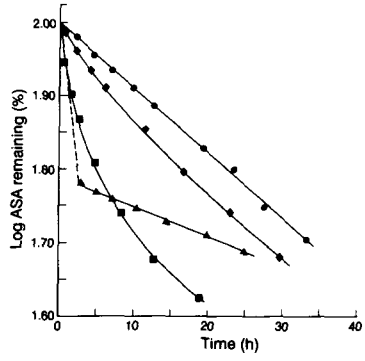


FIG. 2. Kinetics of hydrolysis of ASA at pH 8·0 and 30°C in: ● KH_2PO_4–Na_2HPO_4 buffer solution; ◆ and ■ DMPC:stearylamine liposomes, 2:1 and 1:1 mol ratio, respectively, in which ASA was incorporated via the aqueous phase; ▲ DMPC:stearylamine (2:1 mol ratio) liposomes in which ASA was incorporated via the organic phase.

Table 1. Effect of stearylamine on the stability of ASA in DMPC liposomes at pH 4·0 and 30°C.

DMPC:stearylamine[a]	$k_{obs} \times 10^2$ (h^{-1})	Initial loss of ASA (%)	Increase in stability[b] (%)
2:1	0·800 ± 0·016	34·7 ± 1·15	42·5 ± 1·13
5:1	1·01 ± 0·008	20·9 ± 2·36	27·4 ± 0·56
10:1	1·01 ± 0·009	16·6 ± 2·49	27·4 ± 0·60

[a] Mol ratios; total lipid concentration = 14·4 mM; initial ASA concentration = 7·5 mM. DMPC = dimyristoylphosphatidylcholine.
[b] Using equation 3 and $k_B = 1·39 \pm 0·02 \times 10^{-2}$ h^{-1}; n = 3.

This unusual observation was later found to be due to ASA degradation in the chloroform solution before formation of the lipid film (Fig. 3). However, once the dried film was dispersed in aqueous buffer solution to form liposomes, ASA was better stabilized.

The rapid degradation of ASA in the chloroform solution containing DMPC and stearylamine was essentially completed within 10 min. However, it did not occur in the absence of stearylamine which indicated the possibility of aminolysis, as had been reported for *p*-nitrophenyl acetate (Pejaver & Notari 1985). To confirm this observation, the IR spectra of *N*-stearylacetamide formed from stearylamine-acetic anhydride and from stearylamine-ASA combinations, respectively, were compared and are shown in Fig. 4. There were close similarities in characteristic peaks of N—H at 3300 cm^{-1}, of C—H at ≈3000 cm^{-1}, and of C=O at 1650 cm^{-1}. Furthermore, thin-layer chromatography showed that the R_f value of the reaction product of ASA-stearylamine was identical to that of *N*-stearylacetamide prepared from acetic anhydride.

In Fig. 1, it is evident that at pH 4·0, $k_{obs} = k_B$ in DMPC:stearylamine (2:1) liposomes prepared by method I. In contrast, $k_{obs} < k_B$ following liposome preparation by method II. However, in each case, the rate curve began at a

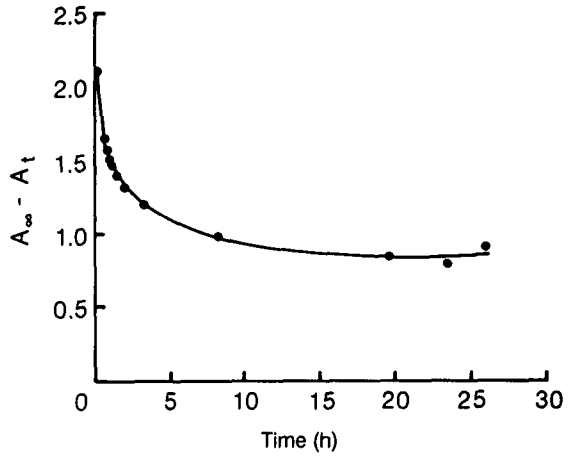


FIG. 3. Hydrolysis of ASA in chloroform solution containing DMPC:stearylamine (2:1 mol ratio) at 30°C. [DMPC] = 14·4 mM; initial [ASA] = 7·5 mM.

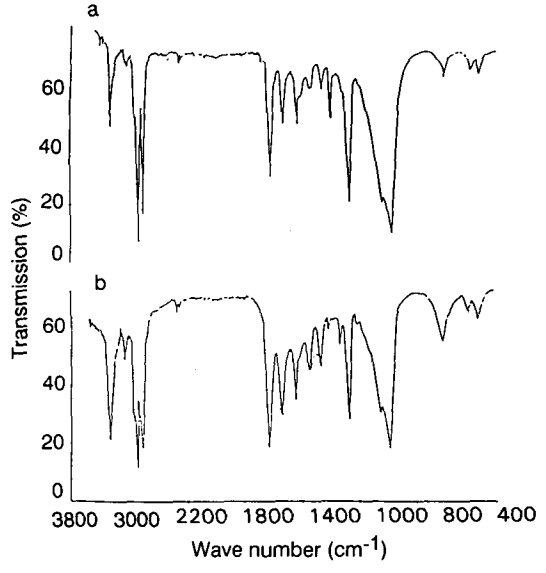
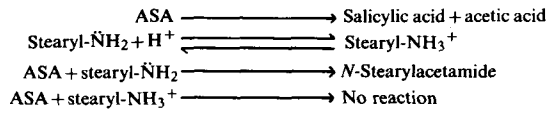


FIG. 4. Infrared spectra of *N*-stearylacetamide synthesized from: a, acetic anhydride and stearylamine; and b, ASA and stearylamine.

lower concentration of ASA. This is because about 35% ASA had degraded by aminolysis before the lipid film was hydrated. In a separate experiment, the kinetics of degradation of ASA associated with the lipid phase only (k_L), was examined by centrifuging, washing, and re-suspending the liposomes. It was found that $k_L \ll k_B$. At pH 8·0 (Fig. 2) a similar pattern of observation was made, except that preparing liposomes by method I the degradation of ASA did not obey first-order kinetics. Also the rate of degradation increased with stearylamine content in the liposomes, although the total lipid was constant. The effect of varying the DMPC:stearylamine mol ratio on the overall degradation of ASA is presented in Table 1. Under identical conditions of liposome preparation, the initial loss of ASA in the solvent phase decreased as the stearylamine content decreased. However, an increase in stability of ASA in the liposomes also decreased to values which were obtained in only DMPC liposomes (Habib & Rogers 1988). These patterns of ASA degradation both at pH 4·0 and 8·0 indicate that for aminolysis to occur, an unprotonated stearylamine molecule should be available so that the lone pair of electrons on the nitrogen atom can react with the ASA molecule.

Loss of ASA in liposomes containing stearylamine can be described by a series of reactions (Pejaver & Notari 1985) shown in scheme 1.



SCHEME 1

Thus, under appropriate pH conditions or in organic solvent, stearylamine behaves as a nucleophile which hydrolyses ASA to form *N*-stearylacetamide.

The degree of stabilization of ASA in liposomes prepared by method II has been attributed to k_L, the rate constant of ASA in the lipid phase, and f_L, the fraction of ASA associated with the lipid phase. Changes in liposome composition mainly affect f_L (Habib & Rogers 1988). Table 2 describes the manner in which additives to DMPC liposomes can affect both the increase in ASA stability and the initial loss of ASA. Thus, the presence of *N*-stearylacetamide did not alter the increase in stability found in DMPC liposomes. Also the addition of cholesterol alone, resulted in the dilution of the stearylamine effect. On the other hand, *p*-chloroaniline (which also posseses a free pair of electrons) exerted an influence similar to that of stearylamine, although having an aromatic ring structure appeared more favourable for reducing the initial loss of ASA and increasing its stability in the liposomes.

The evidence suggests that organic salt bonds are formed between anionic ASA and positive centres of charge within the liposomal bilayers. In DMPC (lecithin) liposomes this occurs with the cationic choline group and also with a cationic amine group if present. However, stabilization of ASA appears to be strongly influenced by its environment. When ASA is added in the aqueous phase at pH 4·0, ASA is not stabilized in DMPC or DMPC:stearylamine liposomes because of interfacial barriers to penetration of the bilayers by anionic ASA to form organic salt bonds. Also, it is not destabilized because the amino or choline groups are still

Table 2. A comparison of the effect of the presence of amines or salicylic acid on the degradation of ASA in DMPC liposomes at pH 4·0 and 30°C.

Liposome composition[a]	Initial loss of ASA (%)[b]	% Increase in ASA stability
DMPC	0	24·5
DMPC:stearylamine (2:1)	34·7	42·5
DMPC:*N*-stearylacetamide (2:1)	0	26·0
DMPC + 2·7 mM salicylic acid (5:1)	0	9·0
DMPC:*N*-stearylacetamide (2:1) + 2·7 mM salicylic acid	0	9·8
DMPC:*p*-chloroaniline (2:1)	27·4	53·0
DMPC:stearylamine:cholesterol	25·0	30·0

[a] DMPC = dimyristoylphosphatidylcholine; total lipid concentration = 14·4 mM; initial ASA concentration = 7·5 mM; mol ratios in parentheses. [b] Degradation of ASA in the organic solvent before liposome formation.

$$ASA^- + \text{lecithin} \xrightarrow{K_A} ASA - \text{lecithin}$$

$$\text{Salicylate} + \text{lecithin} \xrightarrow{K_S} \text{Salicylic acid} - \text{lecithin}$$

SCHEME 2

sufficiently protonated to inhibit the aminolysis reaction according to scheme 1. On the other hand, ASA is destabilized at pH 8·0 (Figs 1, 2) due to its interaction with unprotonated stearylamine or the choline group at the liposome surfaces to form *N*-stearylacetamide or acetyl lecithin (Fatah & Loew 1983). When ASA is added in the organic phase only, stabilization occurs. This may be interpreted to mean that the orientation of ASA molecules, with their centres of reactivity located below the surface of the bilayers, and their ordered structures play an important role in the formation of the organic salt bonds and inhibition of the aminolysis reaction.

Table 2 also includes data showing that salicylic acid has a pronounced effect on the stabilization of ASA. The stabilization of ASA is a function of both k_L and f_L, and the extent of competitive binding of ASA^- and salicylate is described in scheme 2. where K_A and K_S are the association constants of ASA^- and salicylate with DMPC, respectively, and $K_S > K_A$. Similar competitive binding would be expected to occur with stearylamine in the bilayers. Thus, even though ASA-lecithin or ASA-stearylamine offers a stable condition for ASA against hydrolysis, the production of salicylic acid tends to reduce f_L, and hence the overall stabilization of ASA.

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