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The effect of surfactants on the aggregation state of amphotericin B

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We have studied the effect of two surfactants, one non-ionic, lauryl sucrose (LS) and the other ionic, sodium deoxycholate (DOC), on the aggregation state of amphotericin B (AmB) and its selectivity towards ergosterol and cholesterol. It is shown that the addition of these surfactants has very similar effects on the AmB micelles. Below the critical micellar concentration of the surfactants, mixed micelles with AmB are first formed as a result of the penetration of the surfactant molecules into the AmB micelles. At higher concentrations of the surfactant molecules, the micellar structure is completely destroyed and AmB is found as monomers in solution. When the concentration of the surfactant is further increased, micelles of the surfactant molecules are built up, AmB remaining in monomeric form. However, the critical micellar concentration of LS is modified by the presence of AmB in solution, while that of DOC is not affected, thereby indicating that the interactions of AmB with LS are stronger than those of DOC with AmB. We also show that both surfactants enhance the selectivity of the AmB binding to sterols at exactly the concentrations of the surfactants which induce the monomerization of the antibiotic. It is observed that the maximal selectivity is found at a concentration of the surfactants corresponding to their particular CMC in presence of the antibiotic.

Introduction

Amphotericin B (AmB) is the drug of choice for the treatment of systemic mycoses and is becoming increasingly important for use in immunocompromised patients [1]. AmB is relatively toxic to hosts and a number of severe short- or long-term side effects make the treatment difficult, or sometimes even impossible [2]. Therefore, much effort has been recently devoted to the development of a new, less toxic delivery system for AmB. In vitro experiments have demonstrated that in a number of new formulations using lipoproteins [3], lipid emulsions [4], detergents [5] and phospholipid vesicles [6,7], the toxicity of AmB to mammalian cells was significantly decreased without impairing its antifungal activity. In clinical studies liposomal AmB therapy was shown to be less toxic, and as effective as conventional AmB therapy [8].

Numerous studies have shown that surfactants may modulate the activity of drugs. This modulation may be related to changes in drug dissolution, absorption, distribution or binding to cell components [9]. We have recently reported that the cellular toxicity of AmB was increased by low concentrations of a non-ionic surfactant, lauryl sucrose (LS) and decreased by higher concentrations of this compound. The concentrations of LS required to inhibit the antifungal effect of AmB were about 1000-fold higher than the concentrations which inhibited the toxicity to mammalian cells [5,10].

However, the molecular basis of the increased in vitro and in vivo AmB selectivity is still unclear.

Here, we examine the effect of sodium deoxycholate (DOC) and LS on the aggregation state of AmB by light scattering experiments. DOC is the ionic surfactant used in Fungizone, the commercial preparation of AmB and LS is a non ionic surfactant. We report a correlation between the micellar state of these two surfactants and the aggregation state of AmB which may be responsible for the selectivity of AmB to cholesterol and ergosterol and the antifungal activity of the drug [11].

Abbreviations: AmB, amphotericin B; DOC, sodium deoxycholate; LS, lauryl sucrose; CMC, critical micellar concentration.

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Materials and Methods

AmB, DOC, ergosterol and cholesterol were purchased from Sigma (St-Louis, MO). LS was a generous gift of the Mitsubishi-Kasei Food Corporation (Tokyo, Japan). AmB and DOC were used without further purification while ergosterol and cholesterol were twice recrystallized from ethanol (Les Alcools de Commerce Inc., Gatineau, Québec). *n*-Propanol (spectrophotometric grade) was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Phosphate buffer salts were bought from the J.T. Baker Chemical Co. (Phillipsburg, NJ) and used without further purification.

AmB dissolved in DMSO was diluted with water or phosphate buffer to a 5 μmolar stock solution; the final concentration of DMSO was 1%. Stock solutions of LS and DOC were made in water and phosphate buffer at pH 7.4, respectively. All the stock solutions prepared were stored at 4°C. For the solutions involving AmB, the solutions were not kept for more than 24 h prior to use. The solutions of LS, DOC, cholesterol or ergosterol were never kept for more than one week.

The solutions containing AmB, DOC and either ergosterol or cholesterol were prepared from the stock solution of AmB, to which was added the required amount of the DOC stock solution, phosphate buffer, and then the appropriate sterol stock solution. The order of addition of the components is very important in this type of study, the degree of aggregation of AmB being affected by the order of addition. UV-visible spectra were recorded within 30–45 minutes after preparation of the samples. The absorption spectra were recorded using a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. The light scattering signal was measured using a Shimadzu RF-540 spectrofluorimeter, the excitation being at 90°C with respect to the emission signal. Both the excitation and emission wavelengths were fixed at 450 nm. The compounds used did not absorb at this wavelength.

Results and Discussion

The critical micellar concentration of the pure components

We have first measured the critical micellar concentrations (CMC) of AmB, LS and DOC by recording the light scattered at 90°C with respect to the incident line by various concentrations of the compounds. Fig. 1 shows the results obtained for AmB. The results presented are the average of three different measurements done with different solutions of AmB. When micelles are formed in solution, the light scattering signal increases suddenly. The CMC was taken as the concentration corresponding to the intercept of the two essentially linear parts of the plot, i.e. log [AmB] = −6.2, corresponding to a concentration of AmB of $6.3 \cdot 10^{-7}$ M. Absorption spectra recorded for AmB slightly below and above this concentration show that AmB molecules are present, respectively, in monomeric and aggregated forms.

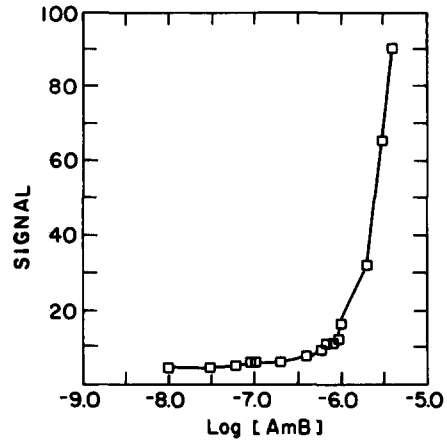

Fig. 1. The critical micellar concentration of AmB in water.

The same general kind of behaviour was observed for DOC and LS. The results obtained for the various pure components are summarized in Table I. The CMC values found for LS and DOC are $6.1 \cdot 10^{-4}$ M and $4.5 \cdot 10^{-3}$ M, respectively. Our results can be compared with those found in the literature, $4.0 \cdot 10^{-4}$ for LS [12] and $(4.0–6.0) \cdot 10^{-3}$ for DOC [13]. It is clear that our experimental determination of the CMC of the pure components gives results very close to those found by other techniques, thereby showing the validity of our method of measurement.

The effect of LS on the aggregation state of AmB

The effect of LS on the aggregation state of AmB was followed by measuring the light scattered by a given AmB solution containing in addition various concentrations of LS. Fig. 2 shows the experimental results obtained for four different concentrations of AmB. One of these concentrations ([AmB] = $1.0 \cdot 10^{-7}$ M, Fig. 2A)

TABLE I

Critical micellar concentrations of AmB, DOC, LS, DOC and LS in the presence of AmB

System	CMC (M)
AmB	$6.0 \cdot 10^{-7}$
LD	$6.3 \cdot 10^{-4}$
DOC	$4.5 \cdot 10^{-3}$
LS in the presence of	
$1.0 \cdot 10^{-7}$ M AmB	$5.8 \cdot 10^{-4}$
$1.5 \cdot 10^{-6}$ M AmB	$5.9 \cdot 10^{-4}$
$6.5 \cdot 10^{-6}$ M AmB	$1.4 \cdot 10^{-3}$
$2.5 \cdot 10^{-5}$ M AmB	$6.3 \cdot 10^{-3}$
DOC in the presence of	
$1.0 \cdot 10^{-7}$ M AmB	$4.5 \cdot 10^{-3}$
$1.5 \cdot 10^{-6}$ M AmB	$4.5 \cdot 10^{-3}$
$6.5 \cdot 10^{-6}$ M AmB	$4.5 \cdot 10^{-3}$
$2.5 \cdot 10^{-5}$ M AmB	$4.0 \cdot 10^{-3}$

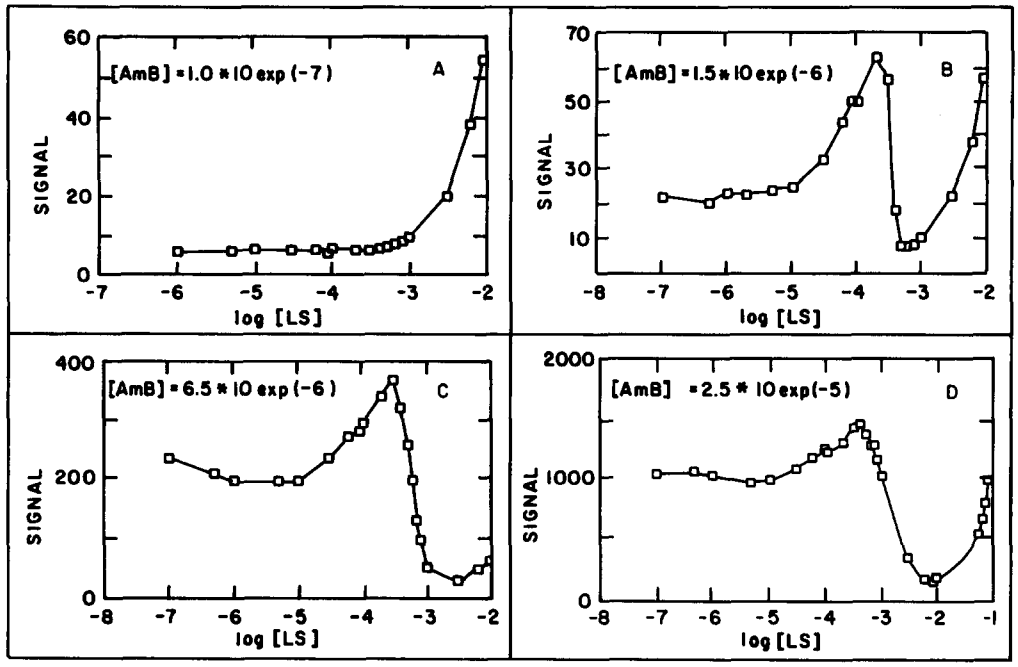

Fig. 2. Effect of LS on the light scattering signal (arbitrary units) of aqueous solutions containing four different concentrations of AmB.

was below the CMC measured for AmB, while the others ([AmB] = $1.5 \cdot 10^{-6}$ M, $6.5 \cdot 10^{-6}$ M, and $2.5 \cdot 10^{-5}$ M, Fig. 2, B–D) were chosen above the CMC of AmB. Fig. 2 shows that the pattern is completely different with the two types of solutions. When AmB is below its CMC (Fig. 2A), the light scattering signal is weak, corresponding to the signal of the pure solvent used. It then starts to increase at log[LS] = −3.2, ([LS] = $6.3 \cdot 10^{-4}$ M, line 4, Table I), i.e., at exactly the CMC of the pure LS. On the other hand, when AmB is above its CMC (Fig. 2, B–D), the light scattering signal first results from the micelles of AmB. Afterwards it is observed to increase above this background level, to then suddenly decrease and reach a minimum value corresponding to the signal measured for the pure solvent. As the concentration of LS is further increased, the light scattering signal starts again to increase.

It is therefore clear that the aggregation state of AmB is indeed modulated by the presence of LS in solution. Thus, when LS is added to micelles of AmB, it appears that the LS molecules penetrate the AmB micelles and change either the size or the form of the micelles,

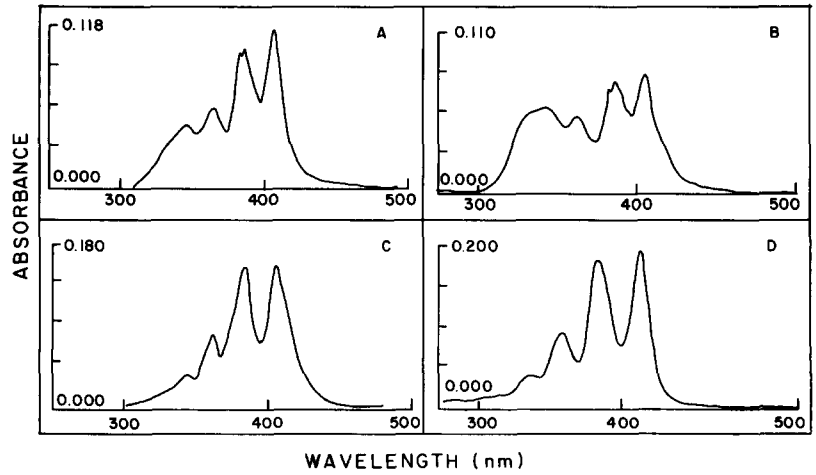

Fig. 3. UV-visible spectra of different AmB/LS mixtures. The concentration of AmB was $1.5 \cdot 10^{-6}$ M and log[LS] was −6.0 (A), −3.7 (B), −3.2 (C) and −2.0 (D).

thereby giving rise to the first increase of the light scattering signal observed (Fig. 2, B–D). Upon further addition of LS, the mixed micellar structure is completely destroyed, as shown by the decrease of the light scattering signal to the pure solvent level. Yet further addition of the detergent gives rise to a new micellar structure, as observed by the increase of the light scattering signal.

Further information about the aggregation state of AmB and its interaction with its environment was obtained by recording absorption spectra of different mixtures of AmB and LS, the spectra of AmB being sensitive to its environment [10,14–16]. Fig. 3 shows typical results obtained for AmB at a constant concentration of $1.5 \cdot 10^{-6}$ M (i.e., above the CMC of this component) in mixtures with LS present at four different concentrations, i.e., log[LS] = −6.0, −3.7, −3.2 and −2.0. These concentrations were chosen to correspond to points typical of the various regions of the light scattering curve shown in Fig. 2B, i.e., before the first increase of the light scattering signal, at the maximum of the signal, at the minimum of the decrease and then in the further rise of the signal.

The first spectrum (Fig. 3A) is typical of a mixture of AmB in both the aggregated and monomeric forms. It shows indeed a narrow maximum of absorption at about 409 nm, characteristic of AmB in monomeric form. Furthermore, the spectrum also shows an absorption band around 328 nm, characteristic of AmB in aggregated form. The spectrum is therefore consistent with the fact that at a concentration of $1.5 \cdot 10^{-6}$ M, close to the CMC of AmB, the two forms of AmB, the free molecules and the molecules in the micelles are contributing to the absorption spectrum. When the concentration of LS is increased to correspond to the maximum in the light scattering plot (log[LS] = −3.7), the spectrum changed as shown in Fig. 3B. The band at 328 nm became much more important than it was before, and correspondingly, the band characteristic of the monomeric form has decreased in intensity. This spectrum leads us to believe that the micelles of AmB have been penetrated by the LS molecules. The modifications of the spectrum observed could arise either from the fact that AmB now interacts with the LS molecules in the mixed micelles or from the fact that the aggregates of AmB get tighter due to the presence of the LS molecules. With further addition of LS (log[LS] = −3.2), the micellar structure is completely destroyed, as shown unambiguously by the light scattering signal (Fig. 2B). The spectrum recorded is now typical of AmB present as free molecules in solution (Fig. 3C). When again the concentration of LS is increased (log[LS] = −2.0), new micelles are formed. The spectrum recorded for AmB in this mixture (Fig. 3D) is similar to the spectrum recorded for the preceding mixture. The nature of these micelles would, however, be different from the micelles formed when lower concentrations of LS were used, as will be discussed below.

The spectroscopic results obtained for the other two concentrations for which AmB is above its CMC (i.e., [AmB] = $6.5 \cdot 10^{-6}$ M and $2.5 \cdot 10^{-5}$ M, Fig. 2, C and D) are in all respects similar to those described above. For these two series of mixtures the spectra recorded are characteristic of the aggregated form of AmB inasmuch as the concentrations of LS are less or equal to the concentration of the mixture for which the light scattered is maximum. Above this concentration of LS, the spectra recorded show that AmB becomes more and more monomerized, to reach a completely monomerized form at the subsequent minimum of the light scattering signal. On the other hand, when the initial concentration of AmB is below its CMC (Fig. 2A), the spectra recorded show that AmB is monomeric whether LS is below or above its CMC.

It is not easy, from the results presented so far, to have a definite position about the location of AmB when the LS micelles are formed at sufficiently high concentrations. Is AmB present as monomers in solution or is it present as monomers in the LS micelles? The fact that the absorption spectra are similar whether the LS micelles are present or not suggests that the molecular environment around the AmB molecules is identical in the two cases, taking into account the fact that the spectrum of AmB is quite sensitive to its molecular environment. This could suggest that even for concentrations of AmB as high as $2.5 \cdot 10^{-5}$ M (i.e., more than one order of magnitude higher than its normal CMC value in pure water), AmB is still present as monomers due to its interactions with the surfactant molecules. Further experiments are now being done to clarify the location of the AmB molecules in the presence of the LS micelles.

As has been shown previously, the toxicity of AmB for mouse erythrocytes and cultured fibroblasts (L-929) was significantly decreased by low concentrations of LS, whereas under the same conditions its toxicity for *Candida albicans* was unaffected [5]. Higher concentrations of LS also decreased the antifungal activity of AmB. Moreover, the concentrations of LS which inhibited the AmB toxicity for mammalian cells enhanced its toxicity for some kinds of fungi [10]. Hence LS enhanced the cellular selectivity of AmB. In these two studies, a good correlation was observed between the cellular experiments and the extent of binding of AmB to cholesterol and ergosterol as monitored spectroscopically. When put in relation with our previous results [5,10] this work shows that the AmB toxicity for mammalian cells and its binding to cholesterol were inhibited in the range of LS concentrations which promoted the AmB monomerization. These concentrations of LS correspond to the minimum of the light scattering signal observed in Fig. 2.

The effect of DOC on the aggregation state of AmB

In order to compare the behaviour of LS with another surfactant, the same kind of study was undertaken with DOC. Fig. 4 shows the light scattering signal obtained from mixtures of AmB and DOC plotted as a functional of log[DOC]. The same four concentrations of AmB as used in the case of the mixtures with LS were used, one of them (Fig. 4A) being under the CMC of AmB. The trend observed for the various concentrations of AmB studied is globally similar to that observed for LS. Indeed, when the concentration of AmB is under its CMC, the light scattering signal corresponds to the value of the pure solvent up to a concentration corresponding to the CMC of pure DOC (Fig. 4A and line 8 of Table I). On the other hand, with the other concentrations of AmB used, the light scattering signal shows a small increase as the concentration of DOC is increased, but not at all comparable to the important increase observed for the LS mixtures. This suggests that the DOC molecules do not seem to penetrate the AmB micelles as effectively as the LS molecules did, thereby indicating that the interactions of AmB with DOC are not as important as those between AmB and LS. However, for the three concentrations of AmB used, an important decrease of the light scattering signal is observed, at concentrations exactly equal to the CMC value of DOC (Fig. 4, B–D, and Table I, lines 9 to 11), suggesting that the AmB micellar structure present up to this concentration of DOC is destroyed. When the concentration of DOC is further increased, micelles are again formed, this time most likely built up mainly from surfactant molecules.

Table I summarizes the CMC values of the pure components and mixtures used in the present study. It clearly shows that the CMC of LS is increased by the presence of increasing concentrations of AmB, the increase being more than one order of magnitude when the concentration of AmB is increased from $1.0 \cdot 10^{-7}$ M to $2.5 \cdot 10^{-5}$ M. On the other hand, the CMC of DOC is not modified by the presence of AmB in solution, at least up to a concentration of $2.5 \cdot 10^{-5}$ M. This striking difference in behaviour points to a difference in the molecular interactions prevailing in solution between AmB and the two surfactants. The results observed do indicate that the affinity of AmB in solution for LS is greater than its affinity for DOC.

The spectra recorded for AmB mixed with DOC present the same general characteristics as those recorded for AmB mixed with LS. Typical spectra are presented in Fig. 5 for a concentration of AmB equal to $1.5 \cdot 10^{-6}$ M, at four concentrations of DOC corresponding to different points of the light scattering data, i.e., below the concentration for which a small increase of the light scattering signal is observed (Fig. 5A) at the maximum (Fig. 5B) and then at the following minimum (Fig. 5C) of the light scattering signal and finally at a higher concentration when DOC micelles are formed (Fig. 5D). For the first two concentrations of DOC, the spectra are characteristic of AmB present as a mixture of aggregates and monomers. At the two higher con-

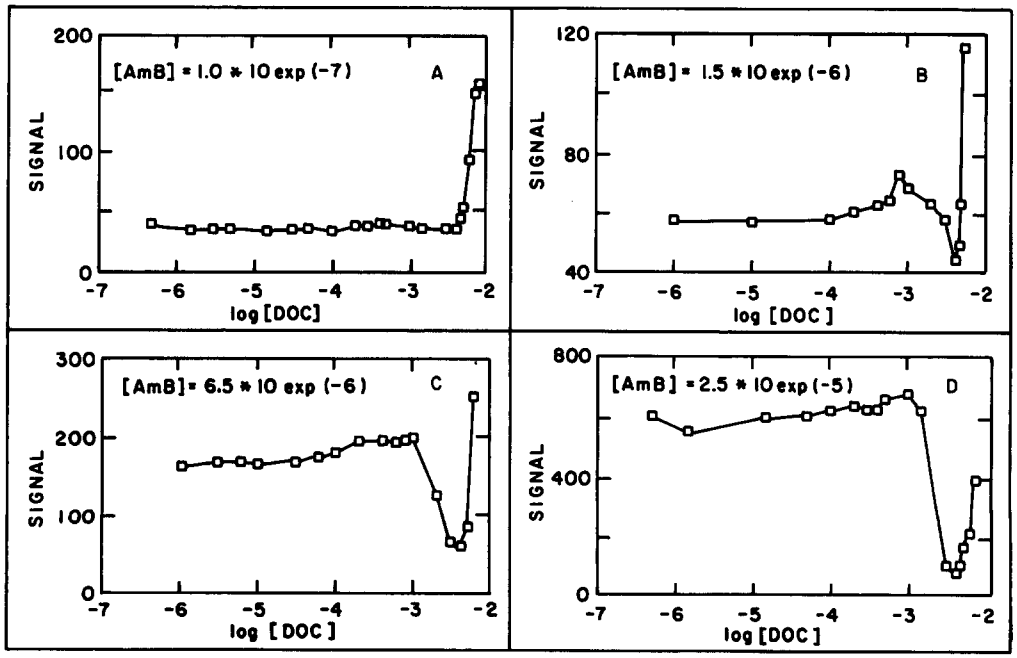

Fig. 4. Effect of DOC on the light scattering signal (arbitrary units) of aqueous solutions containing four different concentrations of AmB.

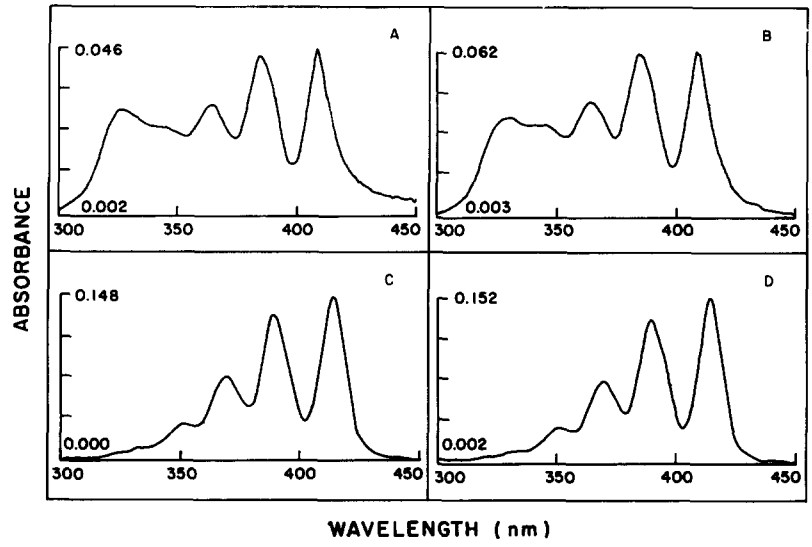

Fig. 5. UV-visible spectra of different AmB/DOC mixtures. The concentration of AmB was $1.5 \cdot 10^{-6}$ M and log[DOC] was −6.0 (A), −3.1 (B), −2.4 (C) and −2.3 (D).

centrations of DOC, AmB is present in monomeric form.

The binding of AmB to ergosterol and cholesterol

In our previous work we determined how increasing concentrations of sucrose esters affect the binding of AmB to ergosterol and cholesterol [5,10]. We used the ratio of absorbance at 409 nm to that at 348 nm ($R = A_{409}/A_{348}$) as a measure of the aggregation state of AmB (in the experiment without sterols) and of its binding to both sterols. We found that the concentrations of LS which caused the monomerization of AmB were also those that induced an increase in the cholesterol-ergosterol selectivity of AmB.

We have used here the same spectral analysis to examine the effect of DOC on the physical state of AmB and its binding to both sterols. Fig. 6 shows the

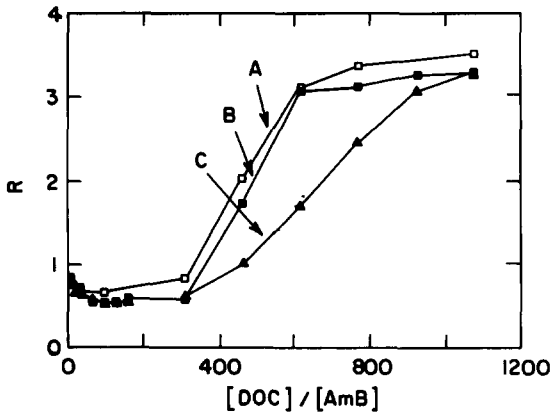

Fig. 6. Effect of DOC on the ratios of absorbance ($R = A_{409}/A_{348}$) of a $6.5 \cdot 10^{-6}$ M AmB solution in the absence (curve A) or presence of equimolar concentrations of cholesterol (curve B) or ergosterol (curve C). The solvent used was a 6.5% solution of propanol in water.

variation of R as a function of increasing concentrations of DOC for AmB alone (curve A) and in the presence of equimolar concentrations of cholesterol (curve B) and ergosterol (curve C). The concentration of AmB chosen for this experiment was $6.5 \cdot 10^{-6}$ M, i.e., above the CMC of this component. It is observed that up to a DOC/AmB molar ratio of about 300, R is not significantly affected by the surfactant, neither for AmB alone nor for the AmB-sterol mixtures. At higher concentrations of DOC for AmB alone an increase in R was observed and a plateau was reached at $R = 3.5$, indicating a shift to the monomeric form of AmB. The DOC/AmB molar ratio at this point was about 600 (i.e., log[DOC] = −2.4), which corresponds precisely to the point where the light scattering signal is at its minimum value (Fig. 4C). Addition of equimolar amounts of cholesterol caused only a slight decrease in R as compared to AmB alone. This shows that the binding of AmB to cholesterol is inhibited in this region of DOC concentrations. Addition of ergosterol lowered significantly R, which indicates that for equal concentrations of the surfactant the extent of binding of AmB to ergosterol is much more important than that to cholesterol.

This DOC induced selectivity of AmB to ergosterol can be quantified by using as a measure $\Delta R = R_{chol} - R_{erg}$. Fig. 7 shows how this parameter was affected by increasing concentrations of DOC. The maximum selectivity was observed at a DOC/AmB molar ratio of 600 (i.e., log[DOC] = −2.4), which, as noted above, corresponded to a solution containing AmB in monomeric form. The cholesterol-ergosterol selectivity decreases at higher DOC concentrations and reaches zero at a a DOC/AmB molar ratio of 1000 corresponding to [DOC] = $6.5 \cdot 10^{-3}$ M, i.e., above its CMC. At this

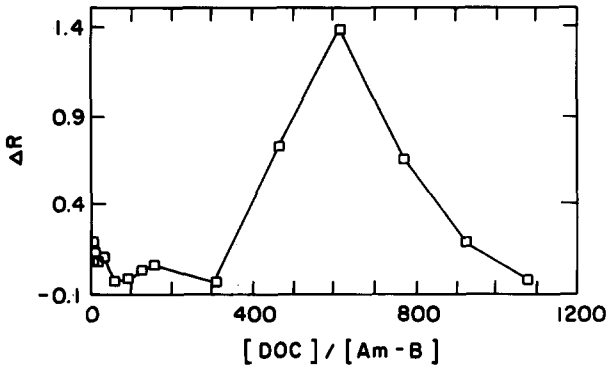

Fig. 7. Cholesterol-ergosterol selectivity of AmB in the presence of DOC as measured by $R_{chol} - R_{ergo}$.

point AmB does not form complexes neither with cholesterol nor with ergosterol. Thus the effect of the increased selectivity of AmB to sterols has the same pattern in the presence of DOC as in the presence of sucrose esters. The only difference is the surfactant concentration needed to induce the selectivity. As DOC has a quite different structure than sucrose esters, in the polar as well as in the lipophilic parts of the molecules, our observations seem to reflect a general feature of the effect of surfactants on the aggregation state of AmB and, consequently, on its affinity to sterols. However, other experimental results with other surfactants will be necessary to confirm this hypothesis.

Recent work on the use of liposomes as delivery system for AmB has shown that the anticellular effects of AmB could be attributed to the levels of AmB remaining free (unbound to the liposomes) in solution [17,18]. These authors show that when only monomeric AmB is present in solution (their so-called 'purified liposomal AmB'), the preparation is not toxic to erythrocytes [17]. In this sense, their work is in agreement with the hypothesis conveyed in the present paper that the selective toxicity of AmB in different delivery systems is related to the presence of monomeric AmB in the system used.

In conclusion, the present work has shown that the addition of two different surfactants, one ionic and the other non-ionic, has very similar dual effects on the AmB micelles. Below the CMC of the surfactants, mixed micelles with AmB are first formed as a result of the penetration of the surfactant molecules into the AmB micelles. At higher concentrations of the surfactant molecules the micellar structure is completely destroyed and AmB is found as monomers in solution. When the concentration of the surfactant is further increased, micelles of the surfactant molecules are built up, AmB remaining in monomeric form. However, the CMC of LS is modified by the presence of AmB, while that of DOC is not affected, thereby indicating that the interactions of AmB with LS in solution are stronger than those of DOC with AmB. We have also shown that both surfactants enhance the selectivity of the AmB binding to sterols at exactly the concentrations of the surfactants which induce the monomerization of the antibiotic. In fact, the maximal selectivity is found at a concentration of the surfactants corresponding to their particular CMC in presence of the antibiotic.

Acknowledgements

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