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Molecular organisation of amphotericin B at the air–water interface in the presence of sterols: a monolayer study

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Abstract

Using the monolayer technique to study the surface behaviour of systems consisting of amphotericin B (AmB) and various sterols, the components were found to interact with each other. The interactions observed are accounted for by postulating that, at low surface pressures, AmB and different sterols form mixed films where the former lies parallel and the latter normal to the air–water interface in such a way that the polar groups in both components establish hydrogen bonds that lead to the formation of an AmB–sterol 'complex' of 2:1 stoichiometry at the interface. At high surface pressures, AmB molecules rearrange themselves normal to the interface; this gives rise to the Van der Waals interactions between non-polar chains of both components that vary with the nature and composition of the system. The occurrence of these hydrophobic interactions prevents the desorption of AmB into the subphase, which is consistent with the positive excess areas of mixing obtained under these surface pressure conditions. Among the four sterols studied, ergosterol exhibits the strongest interaction with AmB and β-sitosterol the weakest. Cholesterol and stigmasterol show intermediate behaviour.

Keywords: Amphotericin B; Mixed monolayer; Sterol film; Interaction at air-water interface

1. Introduction

Amphotericin B (AmB) and nystatin are two polyene antibiotics with major antifungal properties [1,2] probably due to their acting on fungal cell membranes to form internal pores or channels [3] via which crucial elements, such as potassium and various small organic substances (e.g. amino acids and purines), are transferred out of cells causing eventually their lysis and death [4–6]. Membrane sterols seemingly play a highly active role in the formation of these pores; in fact, all types of organisms sensitive to these polyenes (viz. fungi, protozoa, higher algae, mammalian erythrocytes) contain sterols, whereas those insensitive to them (e.g. viruses, bacteria) do not [7].

The necessity of the presence of sterols in cell membranes has been explained on the basis of two different mechanisms. In the first one, the action of sterols is indirect because of their modulate membrane organisation via their ability to alter their phospholipid packing and facilitate the penetration of polyene molecules [8–10]. The other, more widely accepted mechanism postulates the presence of AmB–sterol complexes responsible for the formation of channels composed of alternate molecules of both

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components in the membrane [11–13]. Reported stoichiometry for these hypothetical complexes varies from 1:4 to 1:0.7, depending on the particular method used. Also, the selectivity of polyene antibiotics for plasma membranes has been related to the particular sterol present; in fact, the antibiotics are much more sensitive to ergosterol-rich than to cholesterol-rich membranes [7].

Basing on the above mentioned facts and on the finding that AmB forms stable monolayer when spread on the water surface [14], similarly to sterols [15], the monolayer technique could be an effective means for answering the previous questions. In this work, we studied the surface behaviour of systems consisting of AmB and various sterols (ergosterol, cholesterol, stigmasterol and β-sitosterol) spread at the air–water interface to form mixed monolayers the components of which were found to interact similarly as in cell membranes.

2. Materials and methods

AmB was supplied in 94.7% purity by Bristol-Myers Squibb. Sterols were purchased from Fluka (Germany); cholesterol and ergosterol were of 99% purity, and stigmasterol and β-sitosterol of 98%. None of these substances was further purified since very often the treatments involved introduce contamination by surfactants, which are much more difficult to remove from laboratory assemblies than from industrial synthetic processes.

Spreading solutions were prepared in a solvent consisting of 3:1 v/v dimethylformamide and 1 M HCl. This mixture dissolves AmB, which is insoluble in most of the organic solvents typically used to prepare monolayer spreading solutions. The solutions were stored refrigerated in the dark and were never used for more than 2–3 days. A Microman–Gilson microsyringe was used to deposit a fixed number of amphotericin B and sterol molecules (2.3×10^{16}) on the subphase. This allowed the π–A curves for the different mixtures to be plotted on the same graph without the necessity to alter the x-axis to accommodate the average molecular areas occupied by the mixed monolayers, which were identical for all the mixtures.

π–A curves were obtained by using a Lauda FRG FW-1 surface balance furnished with a Teflon trough with a free surface of 562 cm^2 for spreading the monolayer. The moving barrier was compressed at a rate of 0.11 cm/s (99 cm^2/min); previously, the isotherms obtained over the range 0.11–0.015 cm/s were found to be similar irrespectively to the compression rate used.

The de-ionised water used was obtained from a Milli-Ro, Milli Q system from Millipore (Bedford, MA), which provides 'reagent-grade' water with a resistivity of ca. 18 MΩ/cm. The subphase was kept at constant temperature of 20°C by circulating thermostated water from a Grant LC10 thermocirculator through a channel system located in the bottom of the Teflon trough.

3. Results

3.1. π–A isotherms for amphotericin B-sterol mixed monolayers

Fig. 1 shows the π–A isotherms for monolayers of ergosterol (curve 1) and AmB (curve 7), as well as their mixtures in different proportions (curves 2–6). While ergosterol forms a condensed monolayer typical of sterols [15–20], AmB gives the π–A isotherm with three distinct regions, namely: one at low surface pressures corresponding to an expanded liquid monolayer (compressional modulus 50 mN/m); a plateau of nearly constant surface pressure typical of phase transition; and a third region, where surface pressure increases abruptly as the monolayer is compressed, consistent with the presence of a condensed liquid phase (compressional modulus 104.2 mN/m).

The shape of the π–A isotherm for the mixture containing AmB of 0.9 mole fraction (curve 6) is similar to that for pure AmB (curve 7) except for slightly different collapse pressures and the smaller area of the mixed film relative to the monolayer of pure AmB. Similar comments can be made on the isotherm for the mixture containing AmB of 0.1 mole fraction (curve 2) in relation to that for pure ergosterol (curve 1). In this case, however, the monolayer of the mixture of $X_a = 0.1$ occupies larger area, is more expanded and collapses at higher surface pressure than that of pure ergosterol. In addition, its π–A

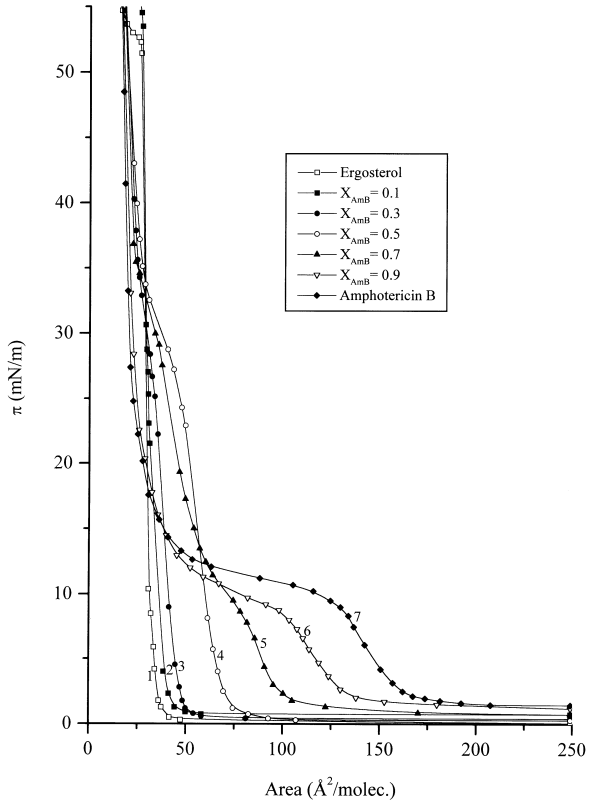

Fig. 1. π–*A* isotherms for AmB–ergosterol mixed monolayers spread on water, at 20°C.

isotherm exhibits a slight inflection at the pressure of 22.5 mN/m.

In any case, the greatest differences in relation to pure components were exhibited by the mixtures of AmB mole fractions between 0.3 and 0.5 (curves 3 and 4), where phase transition occurred at surface pressures in the range of 25–30 mN/m and thus well over the value for pure AmB (10 mN/m). The mixture of $X_a = 0.7$ gives a monolayer that behaves midway between the films of $X_a = 0.5$ and $X_a = 0.9$; its π–*A* isotherm (curve 5) exhibits two inflections (at 10 and 30 mN/m) rather than a single one. The different shapes of curves 3, 4 and 5 in Fig. 1 relative to those for the pure components (AmB and ergosterol, curves 7 and 1, respectively) reveals the existence of strong interaction between them.

AmB–cholesterol mixture of $X_a = 0.7$ gives a monolayer the behaviour of which departs from those of pure components. In fact, as it can be seen from Fig. 2, its π–*A* isotherm (curve 5) has a distinct shape with a single transition zone that starts at a pressure of 21 mN/m and separates two phases of different compressibility: an expanded phase at surface pressures below that of the plateau, with a compressional modulus of 38 mN/m; and a condensed phase above the plateau, with a compressional modulus of 100 mN/m. The mixture of $X_a = 0.5$ gives a film that behaves midway between the previous one and the following mixed films; its π–*A* isotherm (curve 4) contains two inflections at 8 and 25 mN/m, respectively. Similarly, the mixture of $X_a = 0.3$ exhibits two inflection points (curve 3), even though the second (32.5 mN/m) is virtually imperceptible.

The π–*A* isotherms for the other mixtures studied exhibit a plateau of nearly constant pressure (8-10 mN/m) the length of which decreases with decreasing proportion of AmB in the mixed film. On the other hand, the collapse pressure increases with increasing AmB content in the mixture (see Fig. 2).

AmB–stigmasterol system behaves similarly to the

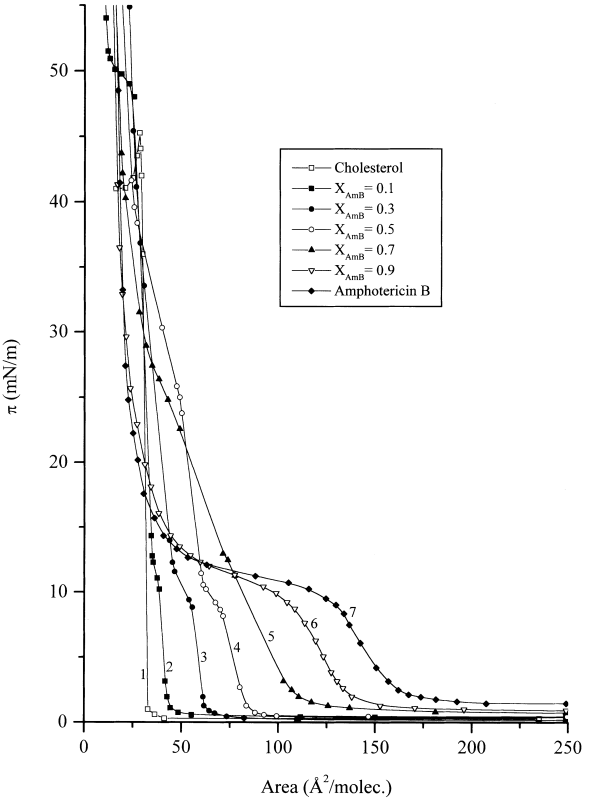

Fig. 2. π–*A* isotherms for AmB–cholesterol mixed monolayers spread on water, at 20°C.

previous one except that it is the mixture of $X_a = 0.5$ rather than that of $X_a = 0.7$ which gives the monolayer most markedly departing from the behaviour of the pure components (see curve 4 in Fig. 3, which exhibits a shoulder at the surface pressure of 25 mN/m that separates an expanded phase and a condensed phase with compressional modulus of 38 and 130 mN/m, respectively). On the other hand, the mixture of $X_a = 0.3$ gives a monolayer the π–A isotherm for which (curve 3) is similar to that for mixture of $X_a = 0.5$ in the AmB–cholesterol system (Fig. 2, curve 4): also exhibits an inflection at 6.0 mN/m and a smooth plateau at 24 mN/m.

The shape of the π–A isotherms for mixed monolayers with high AmB (curves 5 and 6) or high stigmasterol content (curve 2) is similar to those for the pure components (AmB in the former case and the sterol in the latter). Notwithstanding this similarity, there are slight differences between their collapse pressures and other monolayer parameters (see Table 1).

Finally, the behaviour of mixed monolayers consisting of AmB and β-sitosterol is similar to that of pure components. As can be seen from Fig. 4, the shapes of the π–A isotherms are similar to those for the pure sterol when the mixture is rich in this component (curve 2) or to that for AmB when the antibiotic predominates in the mixed monolayer (curve 6). Except for the film of $X_a = 0.1$, which is fully condensed at all surface pressures studied, similarly to film containing pure β-sitosterol, the other mixed monolayers exhibit a transition region at surface pressures between 9.5 and 12.5 mN/m in their isotherms (curves 6 and 3), pressures which are of the same order of magnitude as that for the transition in pure AmB. In these cases, films are in an expanded liquid state below the transition pressure, with com-

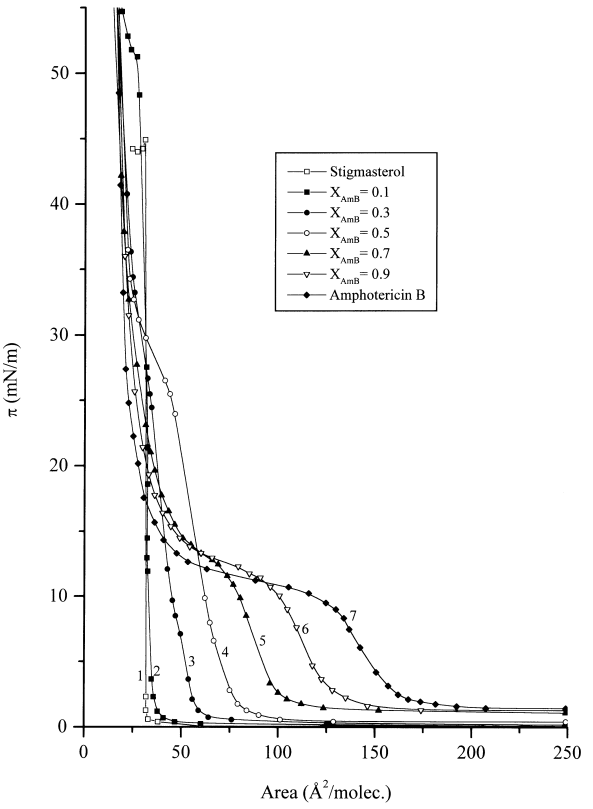

Fig. 3. π–A isotherms for AmB–stigmasterol mixed monolayers spread on water, at 20°C.

Table 1
Transition pressures and collapse pressures for mixed monolayers consisting of AmB and different sterols

Mole fraction of AmB	Ergosterol–amphotericin B		Cholesterol–amphotericin B		Stigmasterol–amphotericin B		β-sistosterol–amphotericin B	
	Transition pressure (mN/m)	Collapse pressure (mN/m)	Transition pressure (mN/m)	Collapse pressure (mN/m)	Transition pressure (mN/m)	Collapse pressure (mN/m)	Transition pressure (mN/m)	Collapse pressure (mN/m)
0	–	51.2	–	45.0	–	45.0	–	48.0
0.1	22.5	53.7	10.0	48.8	–	52.0	–	48.0
0.3	25.0	60.6	9.0 and 32.5	54.3	6.0 and 24.0	55.6	10.0	51.2
0.5	27.5	62.2	8.0 and 25.0	56.3	25.0	59.8	12.5	53.1
0.7	10.0 and 30.0	63.1	21.0	60.0	12.0	63.0	11.5	58.7
0.9	8.6	64.0	10.0	63.8	11.7	64.3	9.5	65.0
1	10.5	65.0	10.5	65.0	10.5	65.0	10.5	65.0

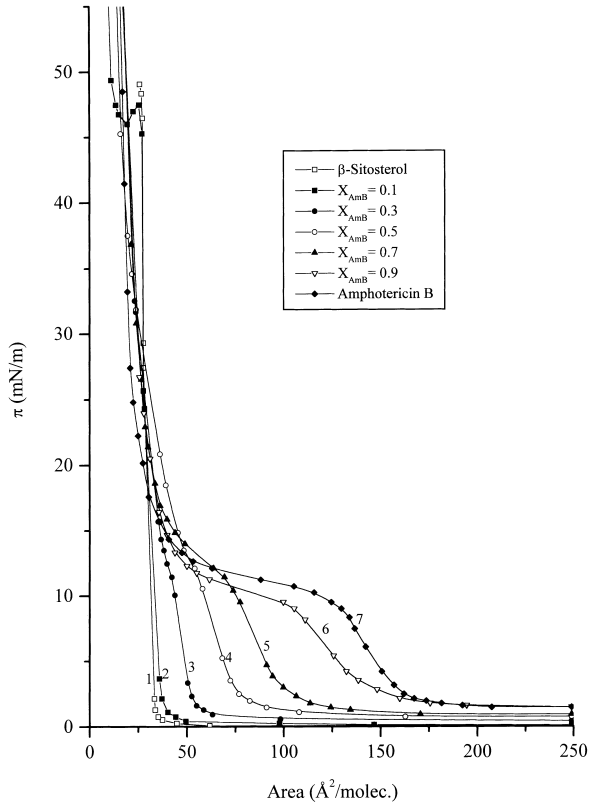

Fig. 4. π–*A* isotherms for AmB–β-sitosterol mixed monolayers spread on water, at 20°C.

pressional moduli of 30–50 mN/m, or in a condensed liquid state above such a surface pressure, with compressional moduli above 100 mN/m. Similarly to the above-described systems, the collapse pressure for the mixed films changes with their composition, from 48 mN/m at $X_a = 0.1$ to 65 mN/m at $X_a = 0.9$ (results not showed).

3.2. Variation of the average molecular area of the mixed films with the amphotericin B mole fraction

The behaviour of AmB–sterol mixed films can also be analysed by checking whether the area they occupy obeys the additivity rule,

$$A_{1,2} = X_1 A_1 + X_2 A_2,$$

where $A_{1,2}$ is the average molecular area occupied by the mixed monolayer at a given surface pressure; X_1 and X_2 are the mole fractions of the mixture components; and A_1 and A_2 are the molecular areas of the pure components 1 and 2, respectively, at the same pressure where $A_{1,2}$ is measured. If the two components are immiscible or ideally miscible, a plot of $A_{1,2}$ against the mole fraction of either component is a straight line; on the other hand, deviations from linearity in the plot suggest miscibility and some type of molecular interaction between the components. Fig. 5 shows such a plot for the four systems studied. As can be seen, all exhibit marked non-linearity. At low surface pressures, below that for the plateau in the π–*A* isotherms, negative deviations are observed throughout the mole ratio range examined. Because all four sterols studied form densely packed, very condensed monolayers, it may safely be assumed that each molecule occupies essentially the same area in both pure and mixed films. However, the AmB monolayer is in an expanded state at low pressure surfaces, where it is highly compressible; this facilitates its 'condensation' by cholesterol, which leads to a contracted area (a partial molecular area) in the mixed film relative to the pure one.

Table 2
Partial molecular areas occupied by AmB and condensing effect produced by sterols in mixed films with AmB

Mole fraction of AmB	Ergosterol–amphotericin B		Cholesterol–amphotericin B		Stigmasterol–amphotericin B		β-Sistosterol–amphotericin B	
	Partial molecular area (Å²/molecule)	Area condensed (Å²/molecule)	Partial molecular area (Å²/molecule)	Area condensed (Å²/molecule)	Partial molecular area (Å²/molecule)	Area condensed (Å²/molecule)	Partial molecular area (Å²/molecule)	Area condensed (Å²/molecule)
0.1	71	75	124	22	69	77	49	97
0.3	71	75	124	22	115	31	99	47
0.5	124	22	124	22	115	31	120	26
0.7	124	22	124	22	127	19	120	26
0.9	131	15	135	11	127	19	146	0

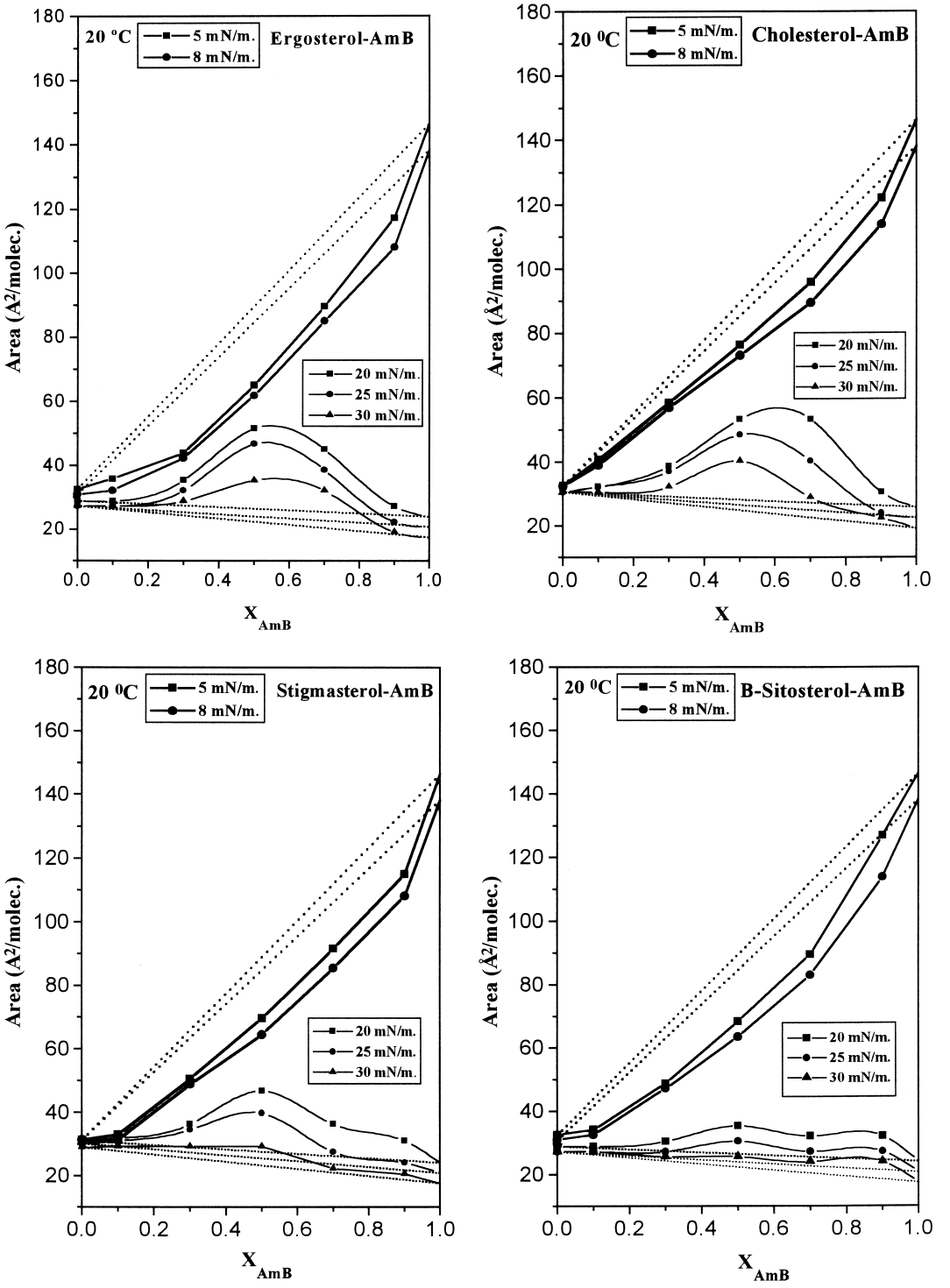

Fig. 5. Plot of average molecular area occupied by AmB–sterol mixed monolayers against amphotericin B mole fraction. pH 6. $t = 20°C$.

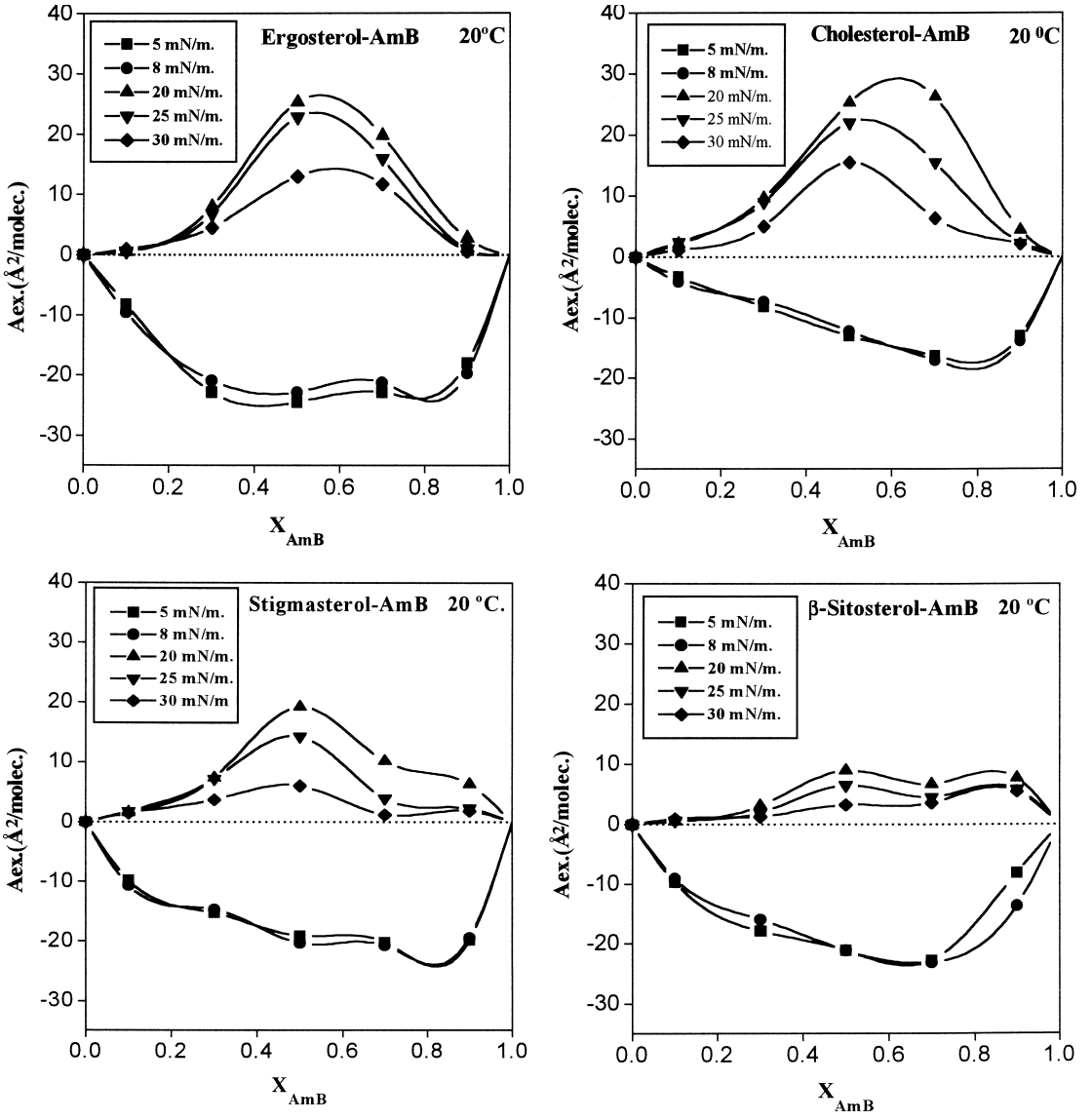

Fig. 6. Plot of excess area of mixing for AmB–sterol mixed monolayers against AmB mole fraction. $t = 20°C$.

Table 2 gives the partial molecular areas for AmB, that is the molecular areas occupied by the AmB molecules in the mixed films at the surface pressure of 5 mN/m:

$$\bar{A}_{\mathrm{AmB}} = A_{1,2} - X_2(\partial A_{1,2}/\partial X_2)_{N_2}$$

where X_2 and N_2 refers to the molar fraction and number of molecules of the sterol constituent in the mixture. At such a surface pressure, pure AmB occupies an area of 146 Å^2 per molecule. The table also includes the values corresponding to the condensing effect of the sterols on AmB. As can be seen, the extent of 'condensation' by the sterols varies with the composition of the mixed monolayer, being particularly significant in sterol-rich films, in which the ergosterol exerts the strongest 'condensing' effect on AmB.

At surface pressures above the phase transition for AmB (20, 25 and 30 mN/m), deviations from linearity in the plot of $A_{1,2}$ vs mole fraction are positive (Fig. 5), and specially marked for the mixtures of $X_a = 0.5$–0.7. The mixtures of AmB with ergosterol and β-sitosterol are those that exhibit the highest and smallest positive deviations, respectively.

3.3. Plots of the excess areas of mixing

Another way of checking the deviations from ideality can be done by plotting the excess areas of mixing (A_{exc}) as a function of the mole fractions of the components. A_{exc} is a quantity defined as the difference between the molecular area occupied by the mixed monolayer at a given surface pressure and the area it would occupy if the components of the mixed film behaved in the ideal manner, i.e.

$$A_{exc} = A_{1,2} - (X_1 A_1 + X_2 A_2)$$

In fact, A_{exc} quantifies the deviation of the mixture components from the ideality. Thus, the larger is A_{exc} (either positive or negative), the more marked deviations will be. The results obtained (Fig. 6) show the existence of negative excess areas in all the systems at a surface pressure of 5 or 8 mN/m, with the largest negative values over the X_a range from 0.3 to 0.7. At surface pressures above the transition value, excess areas of mixing are positive and peak for the mixtures of $X_a = 0.5$ and $X_a = 0.7$. The AmB–β-sitosterol system is that with of smallest positive A_{exc} values.

4. Discussion

4.1. Miscibility of the film components

Most of the π–*A* isotherms for the mixed monolayers studied include a plateau (or inflection point) at a given surface pressure that varies with the composition of the mixed film (see Fig. 7, corresponding to the AmB–ergosterol system). This result could be explained by assuming that, below the surface pressure corresponding to the plateau, the components of the mixed monolayer are immiscible so they behave in the mixed film independently of each another; as a result, the plateau in the π–*A* isotherm could be due to the rearrangement of AmB molecules in the mixed film, from an initial horizontal position to a final vertical one as in its pure monolayers [13,14]. However, applying of the Defay–Crisp phase rule [21,22] to the transition region contradicts this assumption. According to these authors, for a mixed monolayer consisting of *C* insoluble components confined at an interface under constant external pressure and temperature, the Gibbs phase rule takes the form $F = C - P + 1$, where *F* is the number of degrees of freedom of the system and *P* the number of phases involved, air and water excluded. In our case, $C = 2$ (AmB and sterol), so if the mixture components are immiscible at the interface, there will be three phases in equilibrium in the transition region, namely: one consisting of sterol molecules lying normal to the water surface [23]; another consisting of AmB molecules lying horizontally to the water surface; and a third one composed of both, mixed components, with an identical orientation after the transition. Under these conditions, $F = 0$, so the surface pressure corresponding to the phase transition, π_t, must be independent on the system composition. However, the results of Fig. 7 (AmB–ergosterol system) and those of Table 1 (all four systems) reveal that the transition surface pressure varies with composition of the mixed film, which is inconsistent with the assumption that its components are immiscible at the interface. On the contrary, the fact that π_t varies with the composition (so $F = 1$) suggests that the components are miscible and that only two phases exist in equilibrium in the transition region, namely: one consisting of the mixed monolayer M(h-v) formed by AmB molecules parallel to the interface an the corresponding sterol molecules normal to it; and the other consisting of the mixed film M(v-v) formed by the sterol and AmB, both normal to the interface.

On the other hand, the fact that the collapse pressure of the mixed monolayers varies with their composition (Fig. 7 and Table 1) is also consistent with

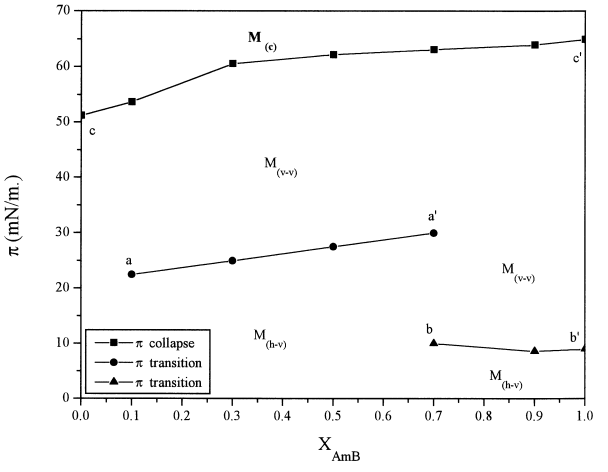

Fig. 7. Plot of transition pressure (lines aa′ and bb′) and collapse pressure (line cc′) as a function of the composition of AmB–sterol monolayers.

the presence of two surface phases in equilibrium that consist of the mixed monolayer M(v-v) with both components normal to the interface, and the collapsed monolayer, M(c), with both components normal to the interface, but collapsed (i.e. as a three-dimensional phase).

4.2. Negative deviations from the additivity rule

According to Saint Pierre-Chazalet et al. [13], these deviations arise from interactions between components in mixed films containing more than 60% of sterol, which cause AmB molecules to arrange themselves normal to the interface, even at low surface pressures; this accounts for the fact that the average molecular area occupied by the mixed film thus formed is smaller than that it would be if it behaved ideally, in which case AmB in the mixture would lie horizontally to the interface as it does in pure monolayers at low surface pressures. However, if this explanation is accepted, the question arises as to what the origin of the plateau in the π–A isotherms for the mixed films is, which was not answered by the authors.

Our explanation relies on the assumption that, at low surface pressures, the mixed monolayer M(h-v), composed of amphotericin B molecules lying horizontally to the air–water interface and sterol molecules vertically to it, is stabilised as consequence of the complex formation with water molecule bridges, via hydrogen bonds, between the sterol 3β-OH group and the carboxyl and amino groups of the sugar residue of the polyene, according to the model postulated by Hervé et al. [24]. Such complexes would occupy a smaller molecular area than the mixed components of ideal behaviour, in which case there would be no additional attractive forces between the components arising from hydrogen bonding.

The results in Figs. 5 and 6 show that the complexes formed are stable within the composition range studied for the mixed films; although the maximum stability correspond to the mixtures of $X_a = 0.5$ and $X_a = 0.7$ in some cases. The presence of complexes of definite stoichiometries or 'specific surface organisations' has been claimed by several authors [25–27]; they postulate that the proportion of each component in the mixed film is critical in relation to the interactions between them as the likelihood of their 'contacting' depends strongly on their 'environment'.

4.3. Positive deviations from the additivity rule

Above the transition region, the mixed films are assumed to consist of AmB and sterol molecules, both lying normal to the interface, so, as they approach each other by effect of compression, hydrophobic interactions are established between the hydrocarbon chains of the polyene and the aromatic rings (together with the alkyl chains) of the sterol. Some authors [24] consider such interactions of extreme significance as they constitute the origin of different behaviour of AmB towards various sterols. Similarly, the differential antifungal activity of different polyene antibiotics has been ascribed to the presence of specific double bounds in their hydrocarbon chains [28].

In any case, these hydrophobic attractions should result in negative deviations from the additivity rule; however, the results obtained (Figs. 5 and 6) reflect the occurrence of positive deviations. According to the reported results [13,14] a plausible explanation for this anomalous behaviour could be that pure amphotericin desorbs into the subphase when it lies normal at the interface (this is supported by the small areas occupied by the monolayer under these conditions); whereas in the presence of a sterol, such desorption takes place in a minor quantity because

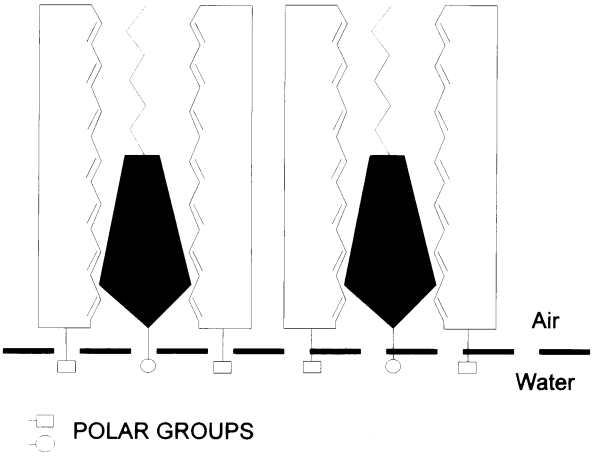

Fig. 8. Arrangement of AmB and cholesterol molecules in monolayers spread on the air–water interface above the transition pressure.

the interaction between both components prevents dissolution of the polyene. Based on this interpretation, the stronger the interaction is, the larger the area occupied by AmB in the mixed monolayers should be relative to that it occupies in its pure monolayer. Because the experimental results for the systems studied show the presence of a maximum in positive deviations for the mixtures with AmB mole fractions of 0.5 and 0.7 (Figs. 5 and 6), and, in accordance with previous hypotheses [13], the maximum stability of the complexes formed between AmB and sterols can be assumed to occur at a 2:1 stoichiometry, which corresponds to the mixture of $X_a = 0.66$. From the energetic point of view, the formation of this complex is highly favoured as it consists of a dimer formed by AmB molecules arranged normally to the air–water interface, with their hydrophobic sides opposing each other, and by sterol molecules jammed between the dimers, interacting with their hydrocarbon chains (Fig. 8).

4.4. Differences due to the nature of the sterol

The presence of the double bond between atoms C_{19} and C_{20} in the terminal hydrocarbon chain of ergosterol and stigmasterol makes their molecules more rigid than those of cholesterol and β-sitosterol. Such a rigidity may be the reason why the negative values of the excess areas of mixing are greater in the former two systems than in the latter (Fig. 6) because the contracted areas result from attractions between the polar groups of the two components; these form a sort of complex of definite composition, the stability of which contributes significantly to increasing the rigidity of the sterol molecule.

On the other hand, the ergosterol structure includes an additional double bond in an aromatic ring (specifically, between its C_7 and C_8 atoms) that can be responsible for the increased condensing effect of ergosterol relative to cholesterol and β-sitosterol. However, the fact that negative excess areas of mixing for mixed films of AmB and stigmasterol are of the same order of magnitude as those for the AmB–ergosterol system rules out a contribution of this condensing effect of the double bond since stigmasterol possesses no such double bond in its aromatic ring.

The positive excess areas of mixing for the mixed monolayers obtained when both film components are arranged normal to the interface are markedly influenced by branching in the terminal hydrocarbon chain of the sterol; the more branched it is, the smaller positive value is. Such is the case of stigmasterol and β-sitosterol, both possessing an ethyl side group in their terminal chain; therefore, in mixed systems with AmB, they give rise to much less marked positive deviations (Figs. 5 and 6) than those exhibited by the systems consisting of AmB and cholesterol or ergosterol. This seems logical taking into account that, as noted earlier, such deviations result from the decreased desorption of AmB molecules in the mixture, which in turn arises from its hydrophobic interaction with the aromatic rings and the terminal hydrocarbon chains of the sterol; the more branched such chains are, the lower the tendency of the sterol being packed among AmB molecules, therefore, will be, which will thus be freely desorbed and lead to a decreased area relative to that it would occupy in the absence of desorption.

Both dependencies: the mean molecular areas and the excess areas of mixing us X_{AmB} suggest that the interactions are stronger with ergosterol; the corresponding parameters have the maximum values of all the systems studied and the interaction was evidenced over the widest composition range (X_a values from 0.1 to 0.7). In this sense, the results confirm the assumption of other authors [24,29,30] that AmB has a greater affinity for ergosterol (a major component of fungal membranes) than for cholesterol (present in animal membranes), explaining these differences its fungicidal action.

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