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## Polydispersity of aggregates formed by the polyene antibiotic amphotericin B and deoxycholate. A spin label study

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The amphotericin B-deoxycholate (AB-DOC) system (1:2, mole basis) was studied with regard to its organizational properties making use of spin label ESR spectra. The spectra of a fatty acid spin label intercalated in AB-DOC preparations revealed two components, one strongly (S) and one weakly (W) immobilized. Spectral subtractions indicated that S corresponds to label in mixed AB-DOC aggregates while W is due to label in deoxycholate micelles. This situation, coexistence of different aggregates, is similar to that found in systems consisting of bile salts and phospholipids. The DOC/AB mole ratio in the mixed aggregate is highest when pure DOC micelles are present. Dilution leads to disappearance of the latter and to continuous loss of DOC from AB-DOC accompanied by an increase in size and decrease in solubility of the aggregates, as verified by filtration and centrifugation experiments. The results indicate that AB-DOC systems are polydisperse. Since amphotericin B preparations having different organizational properties display different toxic and therapeutic effects, the study of amphotericin B aggregates should help in understanding these phenomena at a molecular level.

### Introduction

The polyenic antibiotic amphotericin B (AB) is largely used in the treatment of mycotic infections. It acts at the membrane level, altering cell permeability. Although a large amount of work has been done, the molecular basis for the mechanism of action of amphotericin B is still unclear [1].

Due to its very low water solubility ( $10^{-7}$  M), a preparation containing deoxycholate (DOC) is used in order to solubilize the antibiotic (AB/DOC, 1:2 on a molar basis, Fungizon, [2]). Little is known about the organization of this system at a molecular level. Aggregation phenomena in this preparation have been studied by Rinnert et al. [3] using light-scattering measurements of ethanol-water systems. The optical absorption and

CD spectra of AB-DOC obtained by Rinnert et al. [3] and Ernst et al. [4] were analysed by Hemenger et al. [5], who proposed a helical arrangement for the aggregates.

The use of bile salts to solubilize poorly soluble molecules is a common procedure [6,7]. It has been widely shown that in systems containing bile salts and phospholipids [7–11], there are regions of the phase diagram that correspond to the coexistence of mixed micelles and pure detergent micelles. A theoretical model for this phenomenon has been presented [12].

Previous work has compared the therapeutic and toxic effects of the deoxycholate preparation with those of amphotericin B crystals [13,14] and of its methyl ester [15,16]; different crystals of other antibiotics have also been studied with regard to differences in toxicity and therapeutic effects [17,18]. In addition, recent data on the use of phospholipids [19–23], as carriers for amphotericin B have shown that toxicity is greatly decreased, suggesting that the nature of aggregation affects the toxicity. No definitive explanation is available at present for the different behavior of these preparations.

Here we present a study of the organizational properties of AB-DOC systems, making use of ESR spectroscopy of an intercalated fatty acid spin probe.

Abbreviations: AB, amphotericin B; DOC, sodium deoxycholate; CD, circular dichroism; ESR, electron spin resonance; 5-SASL, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy; ASL, 17 $\beta$ -hydroxy-4',4'-dimethyl spiro(5 $\alpha$ -androstane-3,2'-oxazolidin)-3'-yloxy.

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## Materials and Methods

Fungizone and crystalline amphotericin B (type I) were provided by Squibb Industria Quimica S.A., São Paulo, Brazil, and were stored at 5°C and -15°C, respectively, in the dark. 5-SASL, its methyl ester, ASL, and DOC were purchased from Sigma Chemical Co. All reagents were analytical grade. Doubly distilled deionized water was used throughout.

Amphotericin B suspensions were prepared immediately before use. A concentrated suspension ( $10^{-2}$  M AB) of Fungizone was prepared by adding NaCl to the powder (which already contains sodium phosphates) to obtain a NaCl-37 mM phosphate buffer, 300 mosM. Whenever needed, the pH was adjusted to 7.4. The sample was vortexed for 5 min. Dilutions were done by addition of NaCl-37 mM phosphate (pH 7.4), 300 mosM. DOC samples were prepared in the same buffer system.

Amphotericin B suspensions ( $5 \cdot 10^{-2}$  M, without DOC) were freshly prepared by dissolving crystalline AB in DMSO and diluting to the desired concentration with 300 mosM NaCl-37 mM phosphate buffer (pH 7.4) to a final DMSO concentration of 0.2% (v/v).

Stock solutions of spin labels were prepared in  $\text{CHCl}_3$ , and kept at -15°C. The desired amount of label (1% in moles of amphotericin B, when AB was present, or DOC, in pure DOC preparations) was dried by a stream of nitrogen and kept under vacuum for 2 h. The aqueous preparations (Fungizone, amphotericin B, or DOC) were added, and the sample vortexed for 5 min.

Amphotericin B concentration was determined by measuring the absorbance of samples diluted with DMSO at 415 nm ( $\epsilon = 13 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ).

For filtrations, GSWPO 1300 Millipore filters were used. Centrifugations were done in a Sorvall H-1000 centrifuge, at  $1000 \times g$  for 30 min. ESR spectra were obtained with either Varian E-4 or E-9, X-band spectrometers, at room temperature ( $22 \pm 2^\circ\text{C}$ ), unless otherwise stated. Flat quartz cells for aqueous solutions came from James Scanlon, Costa Mesa, CA. Optical absorption spectra were obtained with a DMR-10 Zeiss spectrophotometer.

## Results

The spin probe 5-SASL was chosen since it seems to be able to intercalate in a non-specific manner in the various aggregates examined in the present work. This is in contrast with the behaviour of its methyl ester which is not incorporated at all, and with that of ASL which, by virtue of its steroid structure, remains always bound to amphotericin B, yielding spectra due to a strongly immobilized probe in all cases (results not shown).

The ESR spectra obtained for 5-SASL in the presence of AB-DOC display two components: one corresponding to a strongly immobilized population (Fig. 1a, S) and another due to a weakly immobilized population (Fig. 1a, W).

Fig. 1 (a-c) shows that, as the preparation is diluted, there is a gradual loss of W. At 1.0 mM amphotericin B, only S is present (Fig. 1c). Computer subtractions of a

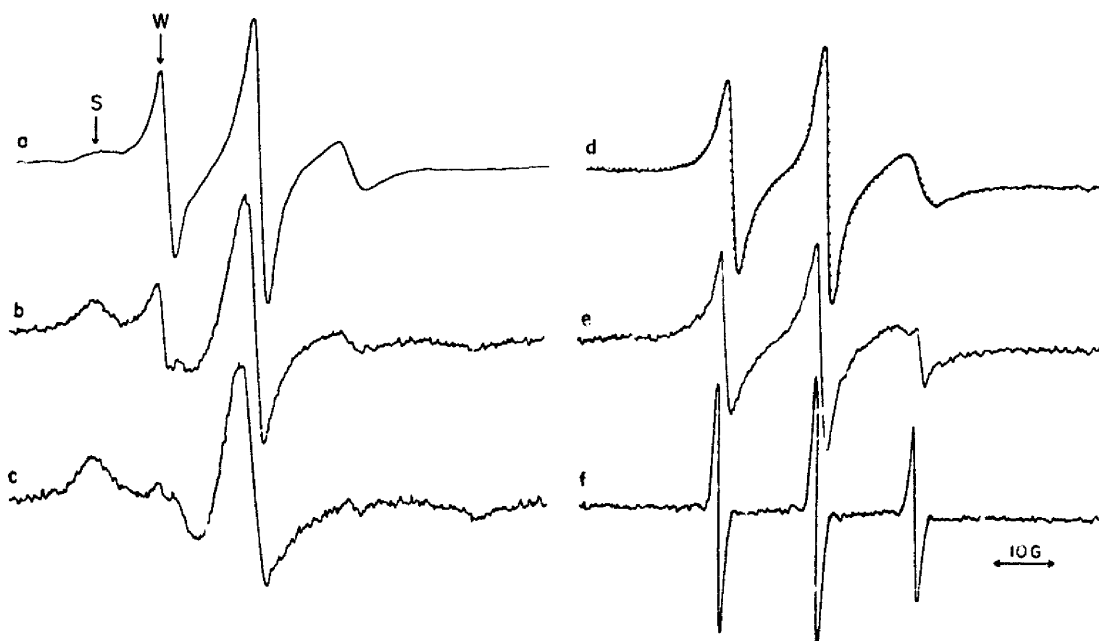


Fig. 1. ESR spectra of 5-SASL in AB-DOC (a-c) and DOC (e-f) systems (see Materials and Methods): (a) 5.0; (b) 1.6; (c) 1.2 mM AB; (d) 6.0; (e) 2.0; (f) 1.0 mM DOC. S and W indicate the low-field components of the spectra due to strongly and weakly immobilized label populations, respectively. The dotted line in d corresponds to the spectrum obtained by subtracting 1c from 1a (for details, see text).

TABLE I

Quantitative analysis of the AB-DOC system as a function of dilution

[AB] (mM)	Total [DOC] (mM)	%W (±5)	[micellar+ monomeric DOC] (mM)	[DOC in the mixed aggregate] (mM)	DOC/AB mol ratio in the mixed aggregates
10.0	20.0	50	6.0	14.0	1.4
5.0	10.0	50	3.0	7.0	1.4
3.0	6.0	40	1.8	4.2	1.4
2.0	4.0	25	1.5	2.5	1.2
1.8	3.6	17	1.5	2.1	1.2
1.6	3.2	11	1.4	1.8	1.1
1.4	2.8	4	1.2	1.6	1.1
1.2	2.4	3	1.2	1.2	1.0
1.0	2.0	0	1.0	1.0	1.0

spectrum of AB-DOC containing 1.0 mM amphotericin B at 24°C (to obtain the same preparation between the outer extrema as that in Fig. 1a, 62.5 G) show that for 5.0 and 10.0 mM antibiotic, S and W contribute 50% each to the total spectrum (Table I); upon dilution, S increases at the expense of W (Table I).

In similar studies with pure DOC, a single spectrum due to weakly immobilized label in detergent micelles is observed for the highest DOC concentrations (3–6 mM, Fig. 1d). As the preparation is diluted, a composite spectrum is observed corresponding to label in DOC micelles and free in solution (Fig. 1e), indicating that 5-SASL partitions between both phases. The component due to 5-SASL in micellar DOC gradually disappears and no more micelles are detected at 1.0 mM DOC; spectrum 1f corresponds to label exclusively in the aqueous phase. Different amounts of spectrum 1f were subtracted from spectra for the higher DOC concentrations to yield the percentage of micelle-bound 5-SASL in each case (Fig. 2). The concentration range in this study is in good agreement with cmc values reported for DOC under similar conditions [24,25].

The spectrum of 5-SASL in pure DOC micelles (Fig. 1d) was compared to that obtained by subtracting pure S (as described above) from the composite spectrum in Fig. 1a. The resulting lineshape (Fig. 1d, dotted line) shows that the match between the two spectra is excellent, indicating that in Fig. 1a, W corresponds to DOC micelles.

In order to check whether S corresponds to mixed AB-DOC aggregates or to pure amphotericin B, we compared S with spectra of 5-SASL in aggregates formed by the antibiotic in the absence of detergent. Pure amphotericin B aggregates gave rise to spectra similar to that in Fig. 1c. However, the separation measured for the outer extrema ( $A_{22}$ ) in Fig. 1c was 63.5 G while that for pure amphotericin B was 66.5 G. This result suggests that spectrum S corresponds to aggregates containing both AB and DOC and that these aggregates

provide a more mobile environment for the spin probe. That this is the case rather than a lower polarity effect was evinced by measurements at  $-140^\circ\text{C}$ , which yielded 68.5 G for the mixed aggregate while amphotericin B alone gave 67.0 G.

In addition, optical absorption spectra of amphotericin B in the two preparations at  $10^{-4}$  M antibiotic are also indicative of differences between pure amphotericin B and AB-DOC systems [26].

Therefore, the antibiotic-bile salt preparations consist of mixed aggregates in equilibrium with pure DOC micelles.

Filtration and centrifugation experiments were performed in order to evaluate the dimensions of the AB-DOC aggregates as a function of concentration. ESR spectra were obtained before and after filtration through a 220 nm filter. It was found that the two-component spectrum at 5.0 mM amphotericin B is totally recovered, whereas the strongly immobilized component is essentially lost at 2.0 mM amphotericin B, and the single spectrum due to the strongly immobilized label is completely lost at 1.0 mM amphotericin B (Fig. 3).

It is noteworthy that although spectra indicative of a high degree of immobilization are observed for all concentrations, the additional filtration procedure demonstrates that they correspond to aggregates of a different size.

Spectrophotometric determinations of amphotericin B in filtrates were in agreement with the above results showing approx. 100% and 0% recovery for 5.0 mM and 1.0 mM amphotericin B, respectively. 2.0 mM amphotericin B samples, however, yielded very scattered results ranging from 20 to 60% recovery.

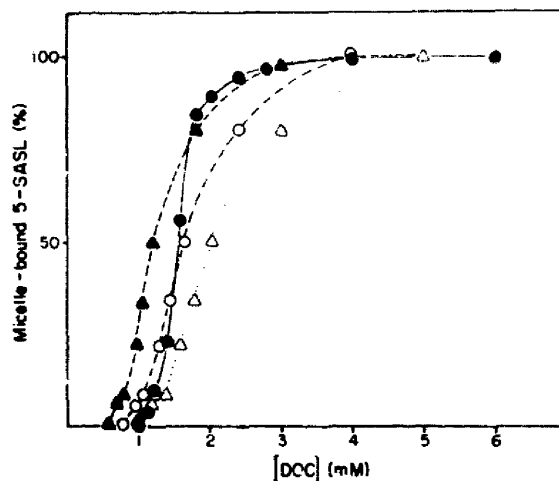


Fig. 2. Calculated percentage of micelle-bound 5-SASL as a function of DOC concentration (●). The values were obtained by subtraction of the spectrum in Fig. 1f from those for higher DOC concentrations. The other curves correspond to the behaviour of 5-SASL in DOC micelles present in AB-DOC mixtures assuming fixed DOC/AB molar ratios in the whole range of AB concentrations: ▲, 1.4; ○, 1.2; △, 1.0. For details, see the Discussion.

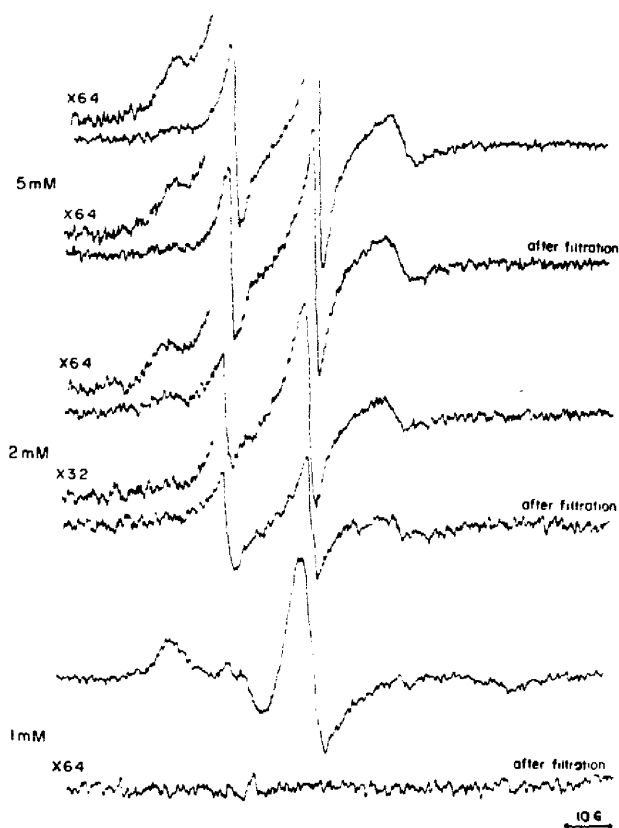


Fig. 3. ESR spectra of 5-SASL in AB-DOC preparations containing 5.0 (top), 2.0 (middle) and 1.0 mM (bottom) amphotericin B, before and after filtration through 220 nm filters. For experimental details, see Materials and Methods.

The centrifugation experiments were in good agreement with the ESR plus filtration data, yielding  $93 \pm 5$ ,  $41 \pm 5$ ,  $16 \pm 3$ ,  $2 \pm 1$  and  $1 \pm 1\%$  amphotericin B recovery for 5.0, 3.0, 2.0, 1.0, and 0.5 mM amphotericin B, respectively (averages of four experiments).

## Discussion

The information concerning the organization of amphotericin B or amphotericin B-deoxycholate aggregates is very scarce [3–5,27].

Rinnert et al. [3] have analysed the organizational properties of AB-DOC systems by light scattering and concluded that the amphotericin B particle was 600 Å (2000 AB molecules) in size. This is a smaller size than found in our studies but it refers to solvent systems at lower ionic strength and containing ethanol, which may have led to smaller particles. No mention is made of the presence of DOC in the aggregates. Considering that DOC micelles might disappear even faster in these systems, and that the study was done at a maximum amphotericin B concentration of  $10^{-4}$  M, little DOC may have actually remained in the aggregates. This would validate the helical model proposed by Hemenger

et al. [5] for aggregated amphotericin B which does not take into account the presence of DOC molecules.

Our results show that the AB-DOC system consists of AB-DOC aggregates coexisting with pure DOC micelles. The spectra of 5-SASL in this system display two components (Fig. 1a): S, corresponding to a strongly immobilized population (AB-DOC aggregates) and W, due to a weakly immobilized population (DOC micelles).

Upon dilution, the spectra indicate that the DOC micelles disappear. The ESR data, in conjunction with the filtration (Fig. 3) and centrifugation results, indicate that the disappearance of DOC micelles is concomitant with an increase in the dimensions and decrease in solubility of the AB-DOC aggregates. The system is polydisperse and consists of particles of the order of thousands of angstroms. The results are in quantitative agreement with the study describing the preparation of Fungizone [2] where it was found that DOC concentrations below 1.5 mg/ml (3.6 mM) are unable to solubilize amphotericin B.

Quasi-elastic light scattering data (Lamy-Freund, M.T., Schreier, S., Peitzsch, R. and Reed, W.F., unpublished data) fully support the above picture.

Table I shows that for the highest amphotericin B concentrations (between 5.0 and 10.0 mM), the spectra correspond to 50% strongly immobilized and 50% weakly immobilized spin label populations. Upon dilution, the decrease in the percentage of W indicates the disappearance of micellar DOC. In contrast to the pure DOC system (Fig. 1d–f), where the spin probe partitioned between the micelles and the aqueous phase, in the AB-DOC system the label lost by the disappearing DOC micelles is incorporated in the remaining AB-DOC aggregates, as indicated by the absence of an aqueous peak and by the increasing conversion of W into S.

The composition of the AB-DOC aggregates and the amount of monomeric and micellar DOC were calculated based on a model that takes into account the above spectral behavior and on the assumption that the DOC micelles in the composite system behave in a similar way to that in pure detergent (Fig. 2).

For the highest amphotericin B concentrations (5.0 and 10.0 mM), W is constant (50% Table I). This would correspond to 100% micelle-bound 5-SASL in pure DOC (100% in the ordinate of Fig. 2). Then, the other values of W (40%, 25%, 17%, and so on) correspond to lower percentages of micelle-bound 5-SASL (80%, 50%, 34%, respectively).

Fig. 2 can be used to obtain the concentrations of micellar + monomeric DOC as a function of dilution, i.e., by using the translated percentages as the ordinates and assuming that the experimental curve holds for the composite system. In Table I, these concentrations are given in the fourth column. The concentrations of DOC in the mixed aggregates (fifth column) were calculated by subtraction of the fourth column from the total

DOC concentration (second column). This analysis shows that the disappearance of micellar DOC leads to a decrease of the DOC/AB mole ratio in the aggregate (last column).

The analysis can be done without uncertainty for 1.2 through to 3.0 mM amphotericin B. For 5.0 and 10.0 mM amphotericin B the following considerations were made: Fig. 2 shows that 3.0 mM is the lowest concentration before DOC micelles start disappearing. Thus, 3.0 mM was taken as the concentration of micellar + monomeric DOC in 5.0 mM amphotericin B, which leaves 7.0 mM DOC in the mixed aggregate (therefore, 1.4 DOC/AB molar ratio). Since the percentage of W remains constant at amphotericin B concentrations  $\geq 5.0$  mM, this would indicate that the DOC/AB ratio also remains constant, i.e., the composition of the mixed aggregate is unaltered above 5.0 mM amphotericin B.

When the percentage of W is 0 we estimate a DOC/AB mole ratio of 1.0 (Table I). This is the lowest possible value consistent with the absence of micellar DOC (see Fig. 2). On the other hand, a higher value would not be in line with the steady decrease of the DOC/AB mole ratio upon dilution.

It is worth noticing that it would not be possible to reproduce the behavior of micellar DOC if the DOC/AB mole ratio were constant throughout dilution. Constant ratios would lead to the simulated curves in Fig. 2.

The AB/DOC mole ratios in Table I are model-dependent. The qualitative conclusion, however, i.e., that the DOC/AB mole ratio decreases upon dilution is granted by the experimental results which indicate that the aggregates become larger and less soluble most likely due to the loss of the solubilizing agent.

Thus, micellar DOC serves as a pool of the detergent to keep the mixed aggregate unaltered (and soluble) according to the following scheme:



and the disappearance of the micelles leads to the continuous loss of DOC from the mixed aggregate.

Our analysis indicates that aggregates containing amphotericin B retain DOC down to the lowest concentrations examined. This conclusion is supported by the different spectra of 5-SASL in AB-DOC and in pure amphotericin B. The spin labeling technique did not allow working at micromolar amphotericin B concentrations such as found in the circulation [28]. It is conceivable that less DOC remains in micromolar amphotericin B, as suggested by the different distribution of DOC and amphotericin B in the body of experimental animals [29].

Bile salts have been extensively studied with respect to their ability to solubilize phospholipids. Except for a few disagreeing reports [30,31], the data have been analysed in terms of the occurrence of a region in the

phase diagrams of these systems where mixed micelles coexist with pure bile salt micelles [7-12]. In the present study it is found that this property is extensive to other solutes.

*Biological implications.* Amphotericin B has very serious toxic effects. Its toxicity and that of other polyene antibiotics seem to depend on the nature of aggregation. Thus, differences in therapeutic efficiency and toxicity have been found between different amphotericin B crystals [14], between crystalline amphotericin B and AB-DOC [13,14], and between AB-DOC and AB's methyl ester [15,16]. In addition, administration of amphotericin B in conjunction with lipoproteins rendered the preparation more toxic [32]. Recent use of phospholipids as carriers of amphotericin B [19-23] has shown that these preparations are considerably less toxic than AB-DOC. Although a definitive explanation for these differences is presently not available, the nature of the aggregates might play a role.

Another aspect concerns the hypothesis that autoxidation of AB might lead to lipid peroxidation processes which would, then, be involved in the mechanism of action of the antibiotic [33,34]. Work from this laboratory has shown that amphotericin B undergoes autoxidation [35]. More recently we have found that the kinetics of autoxidation depends on the aggregation of amphotericin B (Lamy-Freund, Ferreira, Faljoni-Alário and Schreier, in preparation).

A final point refers to the fact that in the circulation amphotericin B is transported by serum proteins (lipoproteins and albumin) [28,36,37]. Nevertheless, as discussed above, the history of the preparation clearly plays a role in its toxic properties. Thus, the equilibria between different aggregates and serum proteins and the kinetics of these processes have to be analysed in order to obtain a deeper insight into the mechanism of action and toxicity of amphotericin B.

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#### References

- 1 Bolard, J. (1986) *Biochim. Biophys. Acta* 864, 257-304.
- 2 Bartner, E., Zinnes, H., Moe, R.A. and Kulesza, J.S. (1957-1958) *Antibiotics Annual*, 53-58.

- 3 Rinnert, H., Thirion, C., Dupont, G. and Lematre, J. (1977) *Biopolymers* 16, 2419-2427.
- 4 Ernst, C., Grange, J., Rinnert, H., Dupont, G. and Lematre, J. (1981) *Biopolymers* 20, 1575-1588.
- 5 Hemenger, R.P., Kaplan, T. and Gray, L.J. (1983) *Biopolymers* 22, 911-918.
- 6 Lichtenberg, D., Robson, R.J. and Dennis, E.A. (1983) *Biochim. Biophys. Acta* 737, 285-304.
- 7 Carey, M.C. (1985) in *Sterols and Bile Salts* (Danielsson, H. and Sjovall, J., eds.), pp. 345-403, Elsevier Science Publishers, Amsterdam.
- 8 Duane, W.C. (1977) *Biochem. Biophys. Res. Commun.* 74, 223-229.
- 9 Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) *Biochemistry* 19, 601-615.
- 10 Higuchi, W.I., Su, C.C., Daabis, N., Wanichsiriroj, A. and Hofmann, A.F. (1984) *J. Colloid Interface Sci.* 98, 9-19.
- 11 Schurtenberger, P. and Lindman, B. (1985) *Biochemistry* 24, 7161-7165.
- 12 Stecker, M.M. and Benedek, G.B. (1984) *J. Phys. Chem.* 88, 6519-6544.
- 13 Hill, G.J., Butler, W.T., Szwed, C.F. and Utz, J.P. (1963) *Am. Rev. Respir. Dis.* 88, 342-346.
- 14 Bennett, J.E., Hill, G.J., Butler, W.T. and Emmons, C.W. (1964) in *Antimicrobial Agents and Chemotherapy*, 1963, pp. 745-752, American Society for Microbiology, Ann Arbor, MI.
- 15 Hoeprich, P.D. (1982) *J. Infect. Dis.* 146, 173-176.
- 16 Kobayashi, G.S., Little, J.R. and Medoff, G. (1985) *Antimicrob. Agents Chemother.* 27, 302-305.
- 17 Ghielmetti, G., Bruzzese, T., Bianchi, C. and Recusani, F. (1976) *J. Pharm. Sci.* 65, 905-907.
- 18 Thomas, A.H., Newland, P. and Sharma, N.R. (1982) *Analyst* 107, 849-854.
- 19 Lopez-Berestein, G., Mehta, R., Hopfer, R.L., Mills, K., Kasi, L., Mehta, K., Fainstein, V., Luna, M., Hersh, E.M. and Juliano, R. (1983) *J. Infect. Dis.* 147, 939-945.
- 20 Juliano, R.L., Grant, C.W.M., Barber, K.R. and Kalp, M.A. (1986) *Mol. Pharmacol.* 31, 1-11.
- 21 Szoka, F.C., Milholland, D. and Barza, M. (1987) *Antimicrob. Agents Chemother.* 31, 421-429.
- 22 Payne, N.I., Cosgrove, R.F., Green, A.P. and Liu, L. (1987) *J. Pharm. Pharmacol.* 39, 24-28.
- 23 Janoff, A.S., Boni, L.T., Popescu, M.C., Minchey, S.R., Cullis, P.R., Madden, T.D., Taraschi, T., Griner, S.M., Shyamsunder, E., Tate, M.W., Mendelsohn, R. and Bonner, D. (1988) *Proc. Natl. Acad. Sci. USA* 85, 6122-6126.
- 24 Benzonana, G. (1969) *Biochim. Biophys. Acta* 176, 836-848.
- 25 Fisher, L. and Oakenfull, D. (1979) *Aust. J. Chem.* 32, 31-39.
- 26 Schreier, S., Ferreira, V.F.N. and Lamy-Freund, M.T. (1985) in *Magnetic Resonance in Biology and Medicine* (Govil, G., Khetrapal, C.L. and Saran, A., eds.), pp. 319-331, Tata McGraw-Hill Publishing Company Ltd., New Delhi.
- 27 Mazerski, J., Bolard, J. and Borowski, E. (1982) *Biochim. Biophys. Acta* 719, 11-17.
- 28 Fields, B.T., Bates, J.H. and Abernathy, R.S. (1970) *Appl. Microbiol.* 19, 955-959.
- 29 Lawrence, R.M., Hoeprich, P.D., Jagdis, F.A., Monji, N., Huston, A.C. and Schaffner, C.P. (1980) *J. Antimicrob. Chemother.* 6, 241-249.
- 30 Claffey, W.J. and Holzbach, R.T. (1981) *Biochemistry* 20, 415-418.
- 31 Muller, K. (1981) *Biochemistry* 20, 404-414.
- 32 Koldin, M.H., Kobayashi, G.S., Brajtburg, J. and Medoff, G. (1985) *Antimicrob. Agents Chemother.* 28, 144-145.
- 33 Brajtburg, J., Elberg, S., Bolard, J., Kobayashi, G.S., Levy, R.A., Ostlund, R.E., Schlessinger, D. and Medoff, G. (1984) *J. Infect. Dis.* 149, 986-997.
- 34 Sokol-Anderson, M.L., Brajtburg, J. and Medoff, G. (1986) *J. Infect. Dis.* 154, 76-83.
- 35 Lamy-Freund, M.T., Ferreira, V.F.N. and Schreier, S. (1985) *J. Antibiot.* 38, 753-757.
- 36 Block, E.R., Bennett, J.E., Livoti, L.G., Klein, W.J., MacGregor, R.R. and Henderson, L. (1974) *Ann. Intern. Med.* 80, 613-617.
- 37 Brajtburg, J., Elberg, S., Schwartz, D.R., Vertut-Croquin, A., Schlessinger, D., Kobayashi, G.S. and Medoff, G. (1985) *Antimicrob. Agents Chemother.* 27, 172-176.