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The role of the carboxyl and amino groups of polyene macrolides in their interactions with sterols and their selective toxicity. A ^{31}P-NMR study

M. Hervé [1,3], J.C. Debouzy [1], E. Borowski [2], B. Cybulska [2] and C.M. Gary-Bobo [1]

[1] *Service de Biophysique, Département de Biologie, CEN / Saclay, Gif-sur-Yvette (France),*
[2] *Department of Pharmaceutical Technology and Biochemistry, Technical University, Gdansk (Poland)*
and [3] *UFR Santé Médecine Biologie Humaine, Université Paris XIII, Bobigny (France)*

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The permeability induced by amphotericin B and vacidin A derivatives in large unilamellar lipidic vesicles containing various sterols has been studied using the proton–cation exchange method and ^{31}P-NMR spectroscopy. Derivatives which have a free ionizable carboxyl group induce biphasic 'all or none' permeability typical of channel-forming ionophores, whatever the sterol present. In sterol-free membranes, they have no significant activity. Derivatives which lack a free ionizable carboxyl group exhibit this channel-like mode of action only in membranes containing ergosterol or sterols with an alkyl side like that of ergosterol. In membranes containing cholesterol or sterol whose side-chain is alike, a slow and progressive permeability is observed at high concentrations. This activity is observed in sterol-free membranes as well. Derivatives containing sugars with substituted amino groups always have lower ionophoric activity than those which are unsubstituted. The greatest decrease in activity was observed for *N*-acetyl derivatives. Substitution of the amino groups has no effect on the mode of action. A model of interaction of polyenes with sterols is presented accounting for the data obtained on vesicles and the observed selective toxicity of polyene derivatives in biological membranes.

Introduction

The selective toxicity of polyene macrolide antibiotics for fungal cells relies on the greater sensitivity to their ionophore action of membranes containing ergosterol than those containing cholesterol [1,2].

From data obtained on both biological and model membranes [3,4] it appears that the amino group of the amino sugar moiety and the C-18 carboxyl group, both constituting the 'polar head' of the macrolide molecule are of primary importance for ionophoric activity.

A comparative study, using ^{31}P-NMR spectroscopy, of this ionophoric activity for various aromatic and non-aromatic polyenes in large unilamellar lipidic vesicles (LUVs), containing either cholesterol or ergosterol, has led to conclusions which are important for the understanding of polyene selective toxicity [5]. Depending on the modifications on the two polar headgroups, polyenes were classified into two groups. Zwitterionic and negatively charged polyenes, which all have a free ionizable carboxyl group, belong to a first group, hereafter denoted group I. They exhibit a poor selectivity, that is a similar efficiency (either high or low) on both cholesterol- and ergosterol-containing membranes. In both membrane types, they induce permeability according to an 'all-or-none' process like gramicidin D, and typical of 'channel-forming' ionophores [6]. The other group, hereafter denoted group II, contains the positively charged polyenes, which all lack a free carboxyl group, this being either absent or substituted (esterified or amidated). These latter polyenes exhibit a qualitatively different behavior depending upon the sterol. In ergosterol-containing membranes they behave like 'channel-formers', but, in cholesterol-containing membranes, they induce a slow and progressive permeability at high concentrations. In consequence, they appear generally less efficient in cholesterol membranes than in ergosterol membranes.

From these results, several questions can be raised. Is it the actual presence of a positive charge which is important, or rather the absence of an ionizable carboxyl group? What is the role of the amino group of the

Correspondence: M. Hervé, Service de Biophysique, Département de Biologie, CEN/Saclay, 91191, Gif-sur-Yvette, France.

amino sugar? Which structural factors in sterol determine the permeabilizing properties of the polyene?

There is a large body of experimental data on the ionophoric properties of polyenes which indicates consistently that these compounds form channel-like structures in association with membrane sterol, and several models have been proposed [7–9] in which amphotericin B forms a 1-to-1 complex with sterol to form a channel spanning the membrane bilayer.

The intrinsic properties of ionic channels formed by various antibiotic molecules have been intensively studied, especially in black films, but few studies have been devoted to antibiotic–membrane lipid interactions. Most generally, the channels are multimolecular assemblies of antibiotics either with themselves or, as it is the case for polyenes, with lipidic membrane components. The channel properties are related to the structure and the stability of these molecular associations in the membrane, which fluctuate between different states. 'Open states' are those in which the molecules properly associated provide a channel exhibiting generally a very high ionic conductance, and 'closed states', those in which either the molecules are dissociated or are associated in non-conducting structures.

Let us consider a cell, or LUV, suspension in which a given concentration of a given polyene is added. The extent of the ionic permeability induced most probably depends primarily on the stability of the sterol-polyene complex, which is assumed to be the basic structure of the open channel.

From this point of view, how crucial is the structure of the polar headgroups of the polyene and their ability to interact through H-bonds with the sterol OH group in the formation of the complex? Group II polyenes show a much higher efficiency in membranes containing ergosterol than in those containing cholesterol. The major difference between the two sterols is in the number of the double bonds. Can these influence the formation of the complex? This would mean that Van der Waals interactions between the hydrophobic moieties of both molecules in the membrane environment could be very important, besides the hydrogen bonding between the polar groups.

In an attempt to answer these questions, the ionophoric properties of a series of modified polyenes, whose biological activity in yeast and red blood cells has been established already [3], has been studied in LUVs by ^{31}P-NMR spectroscopy. Furthermore, experiments have been carried out in LUVs containing sterols differing in the number and location of double bonds, in the presence or absence of a free 3β OH group, and of alkyl substituents in the side-chain, as well as in sterol-free LUVs.

The results of these studies allow the respective role of the carboxyl and amino groups of the polyenes, as well as the role of the sterol structure, to be assessed more precisely. On this basis, a model of sterol–polyene interaction is proposed, accounting for the greater activity in ergosterol-containing membranes of the polyene antibiotics which belong to the so-called group II, i.e., lacking a free carboxyl group.

Materials and Methods

Antibiotics and derivatives

AMB was a Squibb product. Methods of preparation of derivatives have been reported previously [10–12]. The compounds to be tested were dissolved immediately before use in dimethylsulfoxide at a concentration of 1–5 mg/ml and were applied to the vesicular suspension in microliter amounts. FCCP was from Boehringer-Mannheim (Indianapolis, IN) and was used as an ethanolic solution.

Lipids

L-α-Phosphatidylcholine was prepared from egg yolk according to the method of Patel and Sparrow [13]. Phosphatidic acid was enzymatically prepared from it [14]. Cholesterol was from Fluka. Ergosterol, 7-dehydrocholesterol, desmosterol, stigmasterol, cholesteryl chloride and cholesteryl methyl ether were from Sigma Chemical. Brassicasterol was from Research Plus (U.S.A.).

Preparation of vesicles

The preparation of LUVs by reverse-phase evaporation according to the method of Szoka and Papahadjopoulos [15] has been described previously [6]. The lipid composition of LUVs was L-α-phosphatidylcholine/phosphatidic acid/sterol in a molar ratio 80:10:10. For free sterol LUVs, the molar ratio was 90:10. The composition of the aqueous medium was 400 mM sodium phosphate/1 mM EDTA dissolved in 40% ^2H_2O (pH 5.50). After vesicle formation, the suspension was diluted four times in 400 mM sodium sulfate, obtaining a final lipid concentration of 18 mM. Sequentially, the suspension was filtered through polycarbonate porous membranes (Nuclepore Pleasanton, CA) of 1, 0.4 and 0.2 μm pore size. After filtration, the suspension was fairly homogeneous in size (≈ 0.17 μm [16]).

^{31}P-NMR spectroscopy

The pH of 2.5 ml of the filtered vesicle suspension was brought from 5.5 to 7.5 by addition of sodium hydroxide. 10 μl of 1 mM FCCP solution in ethanol were added to provide a non-limiting proton efflux in the presence of antibiotics [6]. The desired amount of antiobiotics was then added. At the end of the desired incubation period, the suspension was transferred into a 10 mm bore NMR tube, together with 20 μl of 100 mM $MnCl_2$ solution, in order to quench the ^{31}P-NMR peak of the external medium. In all spectra shown here, as in

the previous studies [6], the ^{31}P-NMR signals are entirely attributable to the internal phosphate ions (P_{int}), the membrane phosphate signal being negligible at the concentration of lipids used. Conservation of the total signal intensity monitors the integrity of the vesicles.

^{31}P-NMR spectra were recorded at 36.4 MHz with proton noise decoupling on a Bruker WH 90 spectrometer.

Results

Principle of the method

In the presence of a pH gradient between the internal (pH 5.5) and the external (pH 7.5) vesicular medium, the permeability induced by a given ionophore can be measured by the electroneutral proton–cation Na^+/H^+ exchange method described previously [6]. In this method, the cation permeability can be followed by monitoring the proton movement through the membrane either in the external medium by the pH-stat technique [4,6] or in the internal medium by ^{31}P-NMR spectroscopy [5,6].

Using orthophosphate ions as a probe, the chemical shift of the internal phosphate NMR signal reflects the intravesicular pH [17]. Therefore, the monitoring of the internal phosphate peak allows a direct observation of the intravesicular medium on the basis of internal pH. Both the change with time of the position and the intensity of the NMR signal after addition of antibiotic ionophore reflects its activity and its mode of action.

The mobile carrier, valinomycin, induces at low concentrations a progressive broadening and a shift of the initial phosphate signal ($\delta = +0.25$ ppm, pH 5.5) towards its final, equilibrium position ($\delta = 2.2$ ppm, pH 7.5). This shift reflects the equilibration, through the Na^+/H^+ exchange, of the entire LUV population. The proton flux measured by NMR, as well as by the pH-stat method, obeys first-order kinetics [6].

The channel-forming gramicidin D at low concentrations induces a biphasic all-or-none evolution of the phosphate signal: upon gramicidin D addition, the initial phosphate signal immediately splits into two distinct signals. The first one remains centered at $\delta = 0.25$ ppm, its initial position, the second appears at $\delta = 2.2$ ppm, the equilibration value. This second signal increases at the expense of the first. No intermediate signal is detected, and, the first signal, as long as it can be detected, remains at $\delta = 0.25$ ppm [6].

The statistical law of distribution shows that, at low concentrations, a significant fraction of the vesicle population has not enough – or even no – antibiotic molecules in their membrane to induce ion flux. In this condition, the qualitative difference observed in the behavior of the phosphate signal between gramicidin D and valinomycin can be interpreted [6] as the result of the interplay of two kinetics parameters, the intrinsic permeability of the ionophore and its exchange rate between vesicles. The rate of exchange of valinomycin molecules between vesicles is relatively fast compared to the ion flux rate they induce. This latter parameter is the limiting factor of the permeability observed. On the contrary, the rate of exchange of gramicidin D molecule is slow compared to the very high permeability induced by its channels, and therefore the exchange rate is the limiting factor. The only kinetics data obtained by NMR are on the rate of channel formation in vesicles permeabilized in the first place. Due to the ability of the NMR method to distinguish subpopulations of vesicles on the basis of their internal pH, the two modes of action of carrier and channel ionophores may be easily vizualized on the basis of proton flux measurements. However, this distinction can be made only at low ionophore concentrations: a final equilibrium phosphate signal at $\delta = 2.2$ ppm is obtained at high enough concentrations of any antibiotic. As a consequence, the channels are primarily characterized by the observation of an inshifted signal at $\delta = 0.25$ ppm, whereas in the carrier ionophore, the entire initial signal shifts.

This method has been applied to the study of polyene antibiotics [5]. This was made possible because the rate of polyene exchange in LUV is much slower than in SUV [18,19]. It has been shown that amphotericin B methyl ester (AME), as well as vacidin methyl ester (VME), which both belong to group II polyenes, induce a permeability quite comparable to that observed with the gramicidin, i.e., they induce channel-like permeability in ergosterol-containing vesicles only, whereas they induce a carrier-like permeability in cholesterol-containing vesicles. It ought to be recognized that this observation does not mean that the same polyene is able to form channels or to be a mobile carrier depending on the sterol present. As mentioned earlier, the differences detected by NMR spectroscopy refer to kinetic properties which can be very different in ergosterol- and cholesterol-containing membrane – as has been shown by the study of ionic conductance in black lipid membranes [20–22]. This important point will be discussed later.

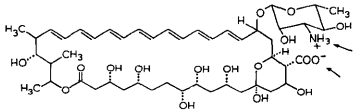
In the present paper, we report the result of such a study on a group of selected polyene derivatives, listed in Table I. Hereafter, these polyenes will be referred to by the abbreviations shown in Table 1.

In all spectra shown, the polyene concentration used is expressed as the antibiotic-to-lipid molar ratio, R. The lipid concentration was the same in all experiments (18 mM). As shown in previous studies [3,4], dose–response curves cover a range of one order of magnitude and the concentration of antibiotics was chosen roughly at the 50% efficiency dose. It was chosen to be high enough to induce a change in the initial spectrum after a 10 min incubation and low enough to avoid equilibrium being reached after the same incubation time.

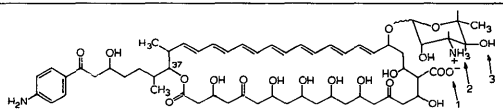
TABLE I

Molecular structures and properties of amphotericin B and derivatives and of aromatic polyenes studied

Amphotericin B



Name	Symbol	Substituent in site		Charge pH 7.5	Group
		1	2		
Amphotericin B	AMB	COO^-	NH_3^+	+−	I
N-Acetylamphotericin B	Ac-AMB	COO^-	$NHCOCH_3$	−	I
Amphotericin B-methyl ester	AME	$COOCH_3$	NH_3^+	+	II
N-Acetylamphotericin B methyl ester	Ac-AME	$COOCH_3$	$NHCOCH_3$	0	II
N,N,N-Trimethyl-amphotericin B methyl ester	DMS-AME	$COOCH_3$	$N^+(CH_3)_3$	+	II
N-(*N'*,*N'*-Dimethylglycyl)-amphotericin B methyl ester	diMeGlyAME	$COOCH_3$	$NHCOCH_2NH(CH_3)_2$	+	II
N-Glycylamphotericin B methyl ester	GlyAME	$COOCH_3$	$NHCOCH_2NH_3^+$	+	II
Amphotericin B-*n*-propylamide	AMBPr	$CONH(CH_2)_2CH_3$	NH_3^+	+	II
Amphotericin B-3-(*N'*,*N*-dimethylamino)propylamine	AMA	$CONH(CH_2)_3NH(CH_3)_2$	NH_3^+	++	II



Vacidin A

Name	Symbol	Substituent in site			Charge pH 7.5	Group
		1	2	3		
Vacidin A	VAC	COO^-	NH_3^+	OH	+−	I
Vacidin methyl ester	VME	$COOCH_3$	NH_3^+	OH	+	II
N,N'-Diacetylvacidin	NAV	COO^-	$NHOOCH_3$	OH	−	I
Perimycin	PE	CH_3	OH	NH_3^+	+	II
N'-Acetylperimycin	NAP	CH_3	OH	$NHCOCH_3$	0	II

Obviously, the equilibrium spectrum (single peak at pH 7.5) would be obtained by addition of any antibiotic and in the presence of any sterol at high enough antibiotic concentration.

Effect of the carboxyl group at C-18

In Figs. 1A–D the spectra obtained in ergosterol- and cholesterol-containing vesicles with AME, Ac-AME, AMBPr and AMA are given pair-wise. These four derivatives of AMB cannot form carboxylate anions because their carboxyl group has been substituted (see Table I). However, they differ by the type of substitution: either by methyl esterification (AME and Ac-AMe), or amidation (AMBPr and AMA). These substitutions are such that the four AMB derivatives differ by their net electric charge: none for Ac-AME, one positive for AME and AMBPr and two positive for AMA. In spite of these differences, all four polyenes exhibit identical behavior. In ergosterol-containing vesicles, upon addition of the polyene, the initial P_{int} signal splits into two separate peaks and, with time, the peak at $\delta = +2.2$ ppm increases at the expense of the initial peak at $\delta = +0.25$ ppm. The disappearance of this initial peak without any shift is a characterization of the 'channel-forming' ionophores. No intermediate signal is observed. After complete equilibration of the system, only one peak at $\delta = +2.20$ ppm is detected. The area included under this peak is equal to the area measured under the initial P_{int} signal before polyene addition. This indicates that during the experiment there is no significant leak of intravesicular phosphate, that is, that no vesicle destruction occurs.

In cholesterol-containing vesicles, the four antibiotics induce a permeability at higher concentration with a

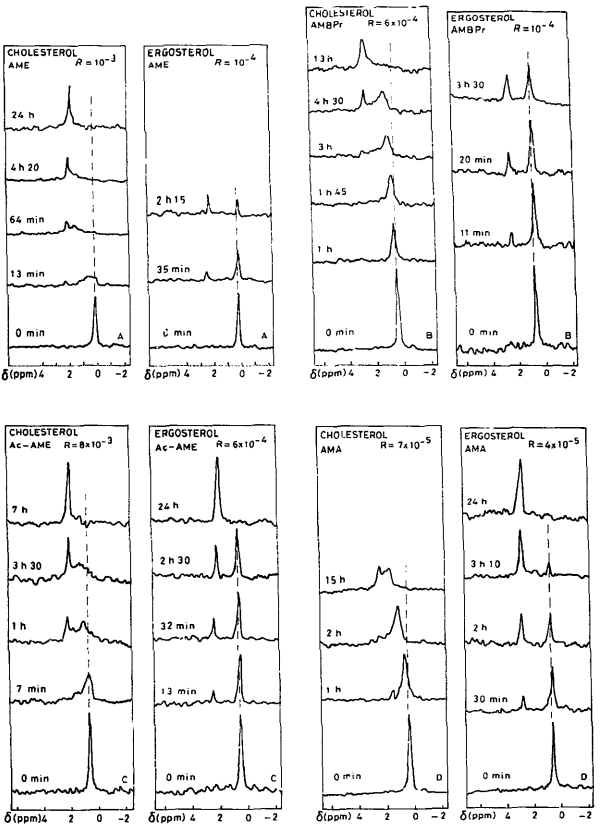


Fig. 1. Evolution as a function of time of the intravesicular phosphate NMR signal in cholesterol- and ergosterol-containing vesicles induced by polyene derivatives having no ionizable carboxyl group. (A) AME, (B) AMBPr, (C) Ac-AME and (D) AMA. *R* indicates the polyene/lipid molar ratio in the vesicle suspension.

different mode of action. Upon addition of the polyene, the evolution of the spectrum is characterized by the broadening of the sharp initial P_{int} signal and its progressive shift towards its equilibrium position. In this case also, the area under the equilibrium peak is equal to the area measured under the initial peak. The same behavior has been observed with all the other AMB derivatives tested which lack a free carboxyl group:

DMSAME, DiMe-Gly-AME, Gly-AME (group II, spectra not shown, but see Table I). Moreover, this has also been observed with aromatic polyenes: VME, PE and NAP (see Table I) [5].

From these and earlier data [5] it is possible to answer the question concerning the role of the carboxyl group.

Firstly, the difference in the mode of action in cholesterol- and ergosterol-containing membranes appears to be a very general characteristic of polyenes of the aromatic and non-aromatic series which lack the free ionizable carboxyl group, whether it is due to a substitution by esterification or amidation, or occurs naturally (PE). On the other hand, all polyenes tested which have a free carboxyl group exhibit the same mode of action pertaining to the 'channel-forming' type, whatever the sterol present in the membrane (results not shown, but see Table I).

Secondly, it appears that the difference in the mode of action in cholesterol- and ergosterol-containing membranes is not related to the electric charge of the molecule. The behavior is the same whether the polyene bears a single charge, two positive charges or no charge (being a non-electrolyte). It can be concluded that it is the lack of the ionizable carboxyl group in itself and not the charge of the molecule which counts.

This leads to a new definition of the two groups proposed above. They were defined by the electric charge of the molecules, and now, they should be defined by the presence (group I) or absence (group II) of a free carboxyl group.

The behavior of these two groups of polyenes in LUVs correlates with their known biological activities [3,4]. The concentration of polyenes of group II necessary to obtain a 50% K^+ leak out of human red blood cells, is systematically 3–5-fold greater than on yeast cells, whereas polyenes of group I are roughly equally active on both cell types. Thus, a clear correlation can be made between the selective toxicity of a polyene and differences in the mode of action in cholesterol- and ergosterol-containing membranes.

Effect of the amino group

As shown above, substitution on the carboxyl group always results in a difference in the mode of action in ergosterol- and cholesterol-containing membranes. Substitution on the amino group of the amino sugar moiety does not have this effect. It only induces an increase in the polyene concentration which is necessary to induce permeability in LUVs, whatever the group type of the polyene.

In the present work, no systematic dose–response curves have been obtained, but from comparisons of the concentrations of the polyenes which were used to observe the development of the permeability process, some conclusions may be drawn.

Considering the efficiency of polyenes of group II in ergosterol-containing membrane, one observes that AME is 6–10-times more efficient than Ac-AME and that PE is 50-times more efficient than NAP. Comparable differences are observed in cholesterol-containing membranes. Likewise, the relationship between efficiency and chemical structure of the amino group is similarly applicable for the first group of polyenes: VAC is 50-times more efficient than NAV in ergosterol-containing vesicles and 10-times more efficient in cholesterol-containing vesicles.

In general, in both groups of polyenes, the greatest efficiency is obtained when the amino group is unsubstituted. The lowest efficiency was observed for *N*-acetyl derivatives in which the protonable amino group was changed into a neutral amide. The efficiency decreases with substitution in the order: $-NH_2 > -NH- \gg NH-CO-$. This observation on LUVs correlates perfectly with the data in biological membranes [3]. For instance, the concentrations necessary to obtain 50% K^+ leak in yeast cells (expressed in $\mu g/ml$ in the suspension medium) are 0.28 for AME, 0.31 for Gly-AME, 1.0 for DMSAME and 10 for Ac-AME. For these four antibiotics, the concentrations necessary to obtain 50% K^+ leak in red blood cells are 0.76, 1.40, 2.70 and 12, respectively. The same observation can be made for the polyenes of group I. The general conclusion is that substitution of the amino group of the amino sugar always results in a drop of efficiency which may reach two orders of magnitude in concentration. The magnitude of the effect seems primarily related to the proton donor ability of the amino group, which is greatest for the unsubstituted amino group. But these substitutions never modify the mode of action, which is governed by the modification on the carboxyl group only.

Effect of sterol

It has been established long ago that the ability to induce ionic permeability requires the presence of sterol in the membrane, and more specifically, sterols having their OH group in the β position. The mode of action of group II polyenes in cholesterol-containing membranes raises the question of whether this requirement is absolutely general. In order to check this point, experiments have been carried out in sterol-free vesicles, and in vesicles containing either cholesteryl chloride or cholesteryl methyl ether, that is, sterols lacking the OH group.

Control experiments of the stability of sterol-free, cholesteryl-chloride or cholesteryl-methyl-containing vesicles were carried out first. The results are given in Fig. 2. These experiments show that in the presence of a 2-unit pH gradient and of the protonophore FCCP but in absence of polyene, the P_{ini} signal shifted very slowly. This shift became significant only after more than 24 h. The whole vesicle population was involved and the

signal, which did not decrease appreciably, scarcely reached its equilibrium position at $\delta = 2.2$ ppm after 3 days or more. This means that in spite of the large proton permeability induced by FCCP, the permeability to Na^+ ion in absence of polyene is practically negligible, as already shown in cholesterol- and ergosterol-containing vesicles [6].

The action of AMB and AME, representative of groups I and II, respectively, has been tested in these vesicles. Typical spectra are shown in Fig. 2.

In the case of AMB, the permeability induced in sterol-free vesicles as well as in vesicles containing cholesteryl methyl ether or chloride is practically negligible. The evolution with respect to time can be distinguished from the evolution observed in control experiments without polyene only at very high concentrations ($R = 10^{-2}$) as shown in Fig. 2. This result is in agreement with observations made long ago by many authors.

In the case of AME, the induced permeability is about the same, at the same concentration, in the three types of vesicle as it was in cholesterol-containing membrane. This result indicates clearly that AME is able to induce ionic permeability without forming complexes with sterol.

Since the only difference between cholesterol and ergosterol is in the number and location of double bonds in the steroid nuclei and alkyl side-chain, we are led to the hypothesis that hydrophobic interaction may be responsible for the differential affinity of polyenes for both sterols.

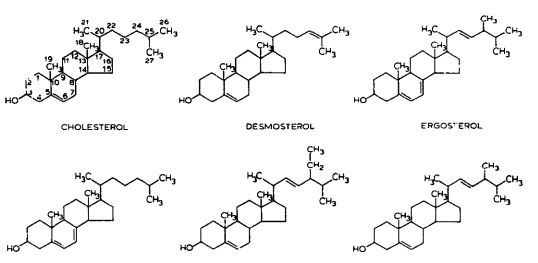
In order to test this hypothesis, the action of AMB and AME was tested in vesicles containing various sterols, whose structures are given in Scheme I.

In the case of AMB, the same 'channel-like' type of ionic permeability is observed whichever the sterol present (spectra not shown, they are all alike, see Fig. 2 of Ref. 5). Only quantitative differences in efficiency are observed, corroborating the results obtained by other authors. In particular, it was already shown on black films [20] that the life-time and frequency of the conducting events induced by AMB depend on the number and position of double bonds in the steroid molecule. All these results indicate that in the case of AMB and polyenes of group I the hydrophilic interaction between the 3β OH group of the sterol and the polar headgroup of the polyenes is the only necessary condition for channel formation, the hydrophobic interaction appearing only as a modulating factor. In the case of AME, the spectra, as expected, depend on the sterol. These spectra are given in Fig. 3, along with those previously shown, obtained with cholesterol and ergosterol, for comparison.

It appears that the spectrum obtained in 7-dehydrocholesterol-containing membranes is similar to the spectrum obtained in cholesterol-containing membranes: AME induces a progressive broadening and shift of the initial P_{int} signal towards the final obtained at equilibrium. However, the spectrum obtained in brassicasterol-containing membranes is identical to that obtained with ergosterol, which is characteristic of a channel-forming ionophore.

In the spectra of desmosterol-containing membranes, a slight shift of the initial peak is detected without significant broadening. In the spectra of stigmasterol-containing membranes, two sharp peaks typical of an all-or-none permeabilizing process are recorded until 2 h incubation, followed by a broadening with shift of the initial peak.

The conclusions which may be drawn from this series of experiments are the following: the presence of a second double bond in the steroid nucleus seems to have no influence on the type of permeability induced by AME; 7-dehydrocholesterol has this second double



Scheme I. Structural formulae of the sterols studied.

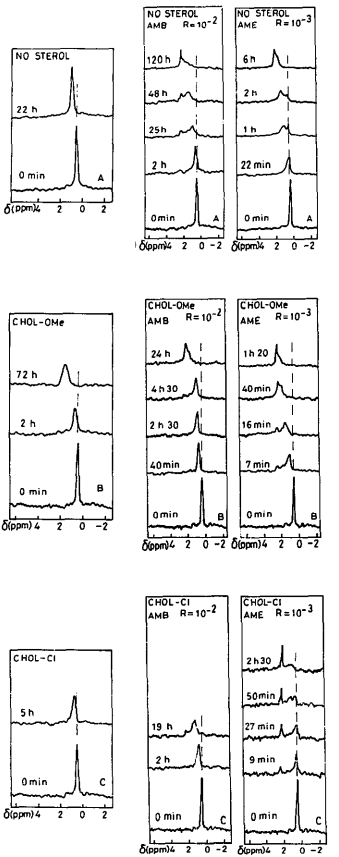


Fig. 2. Evolution as a function of time of the intravesicular phosphate NMR signal induced by AMB and AME in (A) sterol-free vesicles, (B) cholesteryl-methyl-ether-containing vesicles and (C) cholesteryl-chloride-containing vesicles. *R* as in Fig. 1.

bond and the induced permeability is the same as in cholesterol vesicles; brassicasterol does not have this second double bond and the permeability obtained is the same as in ergosterol.

The structure of the alkyl chain seems to be the dominant factor. As shown in Table II, cholesterol and 7-dehydrocholesterol have the same saturated alkyl chain and brassicasterol has the same alkyl chain as ergosterol,

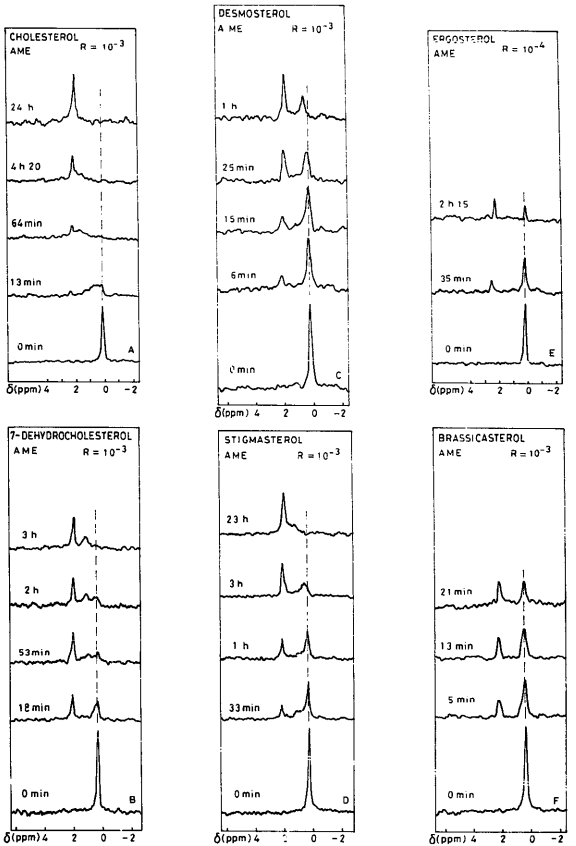


Fig. 3. Evolution as a function of time of the intravesicular phosphate NMR signal induced by AME in vesicles containing various sterols. *R* as in Fig. 1.

with a double bond at the C-22 position. Stigmasterol has the same double bond in the alkyl chain as ergosterol, but with a bulky ethyl group instead of a methyl at the C-24 position. Desmosterol has a double bond, but in the C-24 position. Therefore, it appears that the stability of the AME sterol complex is highly

dependent on the precise structure of the sterol alkyl chain.

The presence of an unsaturated chain seems to be a factor of primary importance for channel-type permeability, but the efficiency of the channels is related to the exact position of the double bond in the chain and the absence of a bulky substituent.

Discussion

The generally accepted mechanism of ionophoric action of the large ring polyene is based on the assumption that these molecules form complexes with sterol. Sterol-polyene complexes which organize themselves in transmembrane channels have been demonstrated only in the case of non-ionophoric small ring polyenes, such as filipin [23]. Unfortunately, in spite of many attempts, it has been impossible to provide any direct evidence of such complexes in the case of amphotericin B or nystatin [2]. For this reason, the different pore models proposed [7–9], which account for most of the known permeability data, are not yet supported by any structural evidence. The present study does not provide such evidence either, but allows a more precise definition of the molecular parameters important for the formation and stability of polyene–sterol channels.

Considering the polyene-sterol association, it must be noted first that sterols already interact with phospholipids, and that there is competition between polyene and phospholipids for sterol in the membrane: this has been shown recently by a comparative study of AMB-sterol and AMB-sterol-phospholipid monolayers [24]. The numerous studies of cholesterol–phospholipid interactions have shown [25,26] that, firstly, the 3β OH group of cholesterol can form a hydrogen bond to the carbonyl oxygen of the *sn*-1-chain of the phospholipid and secondly, the β face of the steroid nucleus, out of which protrude the two angular methyl groups, is in contact with the *sn*-2-fatty acyl chain (generally unsaturated) while the α face is in close contact with the saturated *sn*-1-fatty acyl chain to maximize the Van der Waals contacts.

Although data on ergosterol are scarce, as compared to those on cholesterol, it is reasonable to assume the same pattern of interaction in both cases. However, there is evidence that the ergosterol–phospholipid interaction is weaker than the cholesterol–phospholipid interaction [27]. This might account for the observation that polyenes of both groups are more efficient in ergosterol- than in cholesterol-containing membranes. Even if it is assumed that both sterols interact in the same way with polyenes, the competition with phospholipids would be more favorable in the case of ergosterol.

It may be reasonably assumed that the pattern of interaction between polyene and sterol is similar to the pattern of phospholipid–sterol interaction. It was shown that polyene molecules entering the membrane, most probably as oligomers, align themselves in a position parallel to sterols and phospholipid molecules [28] with their polar heads at the membrane/water interface. In this position, the polyene polar head functional groups may interact by H-bonding with the 3β OH of the sterol. Hydrophobic interactions between the steroid nucleus and its alkyl tail, and the rigid polyenic part of the macrocyclic ring can also be established. Both interactions contribute to the stability of the sterol-polyene complex [29].

The conclusions from this assumption about the sterol–polyene interaction which can be drawn from the present results and from the data previously obtained [5] may be resumed as follows. First, the efficiency of the polyene permeabilizing activity in forming ionic pathways depends on the proton donor ability of the amino group of the amino sugar. This indicates that the hydrophilic interaction must occur through a H-bond between this amino group and the 3β OH of sterol. Two groups of polyene antibiotic can be distinguished according to their ionophoric properties. Polyenes of group I are characterized mainly by the presence of a free ionizable carboxyl group in the C-18 position in the macrolide ring. They all form channel structures of high ionic permeability in sterol-containing membranes, regardless of the structure of the sterol molecule, provided a 3β hydroxyl is present. In sterol-free membranes or in membranes containing sterol lacking in 3β OH, they do not form channels and ionophoric properties are hardly observed.

Polyenes of group II are characterized mainly by the absence of free ionizable carboxyl groups. They all form channel-like structures of high ionic permeability in sterol-containing membranes, but the requirement concerning the sterol structure seems to be very specific. Besides the presence of the 3β OH, a specific structure of the alkyl side-chain, such as that of ergosterol, is necessary. On the other hand, in membranes containing other sterols, as well as in sterol-free membranes, they exhibit quite significant ionophoric properties at relatively high concentrations.

From the functional data, and from the crystal structure data of cholesterol [30,31], ergosterol [32] and AMB [33], a hypothesis can be formulated on the role of polyene–sterol interaction in the mode of action of polyene: the specific antifungal activity of polyenes which is characterized by the absence of free ionizable carboxyl group has been taken into account.

Essentially, a H bond would be established between the protonable amino group of the polyene amino sugar as hydrogen donor, and the sterol's 3β OH as acceptor. For steric reasons, it appears probable that, according to situations observed before [34,35], this H-bond is established through an intermediary water molecule as

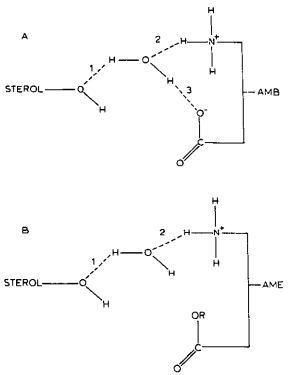


Fig. 4. Schematic representation of the H-bond formation between the polyene derivatives polar headgroups and the sterol OH group in the case of (A) derivatives with free ionizable carboxyl group and (B) derivatives with blocked carboxyl.

depicted in Fig. 4. The H-bond formation depends mainly on the state of the polyenes carboxyl group: in the case of a free and ionized carboxyl group, a strong electrostatic interaction with the amino group maintains the amino sugar moiety in a fixed conformation in space and a complementary binding is established between the carboxylate and the sterol (Fig. 4A). Such a complete H-bond system is very strong and can ensure, by itself, the stability of the polyene-sterol complex. The hydrophobic interactions are of secondary importance. If the carboxyl group is blocked, the amino sugar is free to rotate; the H-bond between the amino group and the sterol is still established, but under less favorable conditions. Furthermore, there is no complementary bonding with the carboxyl (Fig. 4B). Therefore, the H-bond system is relatively weak, and the hydrophobic interactions are of primary importance to the stability of the complex. Since the magnitude of the dispersion forces is extremely dependent upon the distance of closest approach and the matching of hydrophobic surfaces, the specificity of the sterol structure shows up.

A particular structure of the sterol alkyl chain, more precisely the double bond at the C-22 position, appears necessary. Moreover, it is possible that this very structure hinders the interaction of the same sterol with the acyl chain of the phospholipids.

According to the classical channel theory, the ionophoric properties of a channel depend on both its intrinsic ionic permeability and the life-time of its 'open state'. Assuming that polyenes form channels in association with sterols, the strength on this association appears as a predominant factor conditioning their efficiency and eventually their selectivity for membranes containing a specific sterol, as observed in the case of the polyenes of group II.

It remains to be clarified by which mechanism these polyenes are able to induce a significant ionic permeability in the absence of any sterols. The fact that phenomenologically they behave as carriers does not mean that they do so in practice. They may form, by self-association, very unstable channels of low conductance.

Like the previous channels models proposed, the hypothesis made presently on the basis of functional data must find structural support. In order to do so, a structural NMR study of the behavior of ^{13}C-labelled C-3 site of cholesterol has been undertaken in the presence of AMB and AME in LUVs. Preliminary results support our hypothesis somewhat, showing the involvement of the 3β OH group in an interaction in the case of AMB but not in the case of AME. Furthermore, a study of the energetics of polyene–sterol interactions by computer modeling has been undertaken.

The model presented here is mainly based on data obtained on lipid membrane models. The presence of proteins in biological membranes may play a significant role in this sterol–polyene association as shown recently [36]. In any event, there is a good correlation between data obtained in LUVs and biological membranes [3].

Whatever the actual molecular mechanism, it appears that the selective toxicity of polyenes of group II is based not only on quantitative difference in efficiency in ergosterol- and cholesterol-containing membranes but also most probably on a qualitative difference in the mode of action. This provides hope that it will be possible to design better, and much less toxic drugs in antifungal chemotherapy, which are more and more needed in view of the increasing number of immuno-depressed patients today.

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