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# HOW DO IONIC CHANNEL PROPERTIES DEPEND ON THE STRUCTURE OF POLYENE ANTIBIOTIC MOLECULES?

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## Summary

A study has been made of the properties of ionic channels formed in phospholipid-cholesterol bilayers by polyene antibiotics of various molecular structures. Properties of channels created by natural antibiotics with different structures of the lactone ring (amphotericin B-nystatin-mycoheptin) as well as by some derivatives of amphotericin B modified with respect to the amino and carboxyl groups are compared. Neutralization of one or both charges of the amphotericin B molecule (both by chemical modification and by pH shift) increases the probability of the channel to be in a nonconducting state. An increase of cholesterol concentration in the membrane produces an opposite effect. It is assumed that the electrostatic interaction of the amino group of an antibiotic molecule with the carboxyl group of an adjacent one stabilizes the channel. Conductance and selectivity of an open channel are not influenced by changes in the charged groups. These properties strongly depend on the structure of the polar chain of the lactone ring. For example, the appearance of one more carbonyl group in the mycoheptin molecule results in a sharply decreasing anion permeability of channels. An antibiotic concentration which is necessary to observe single channels depends on the polyene chain structure: this is about $10^{-7}$ M for tetraene nystatin and $2 \cdot 10^{-8}$ M for heptaene amphotericin B and mycoheptin.

## Introduction

Polyene antibiotics amphotericin B and nystatin in a complex with sterol form in a lipid membrane pore permeable to ions, water and non-electrolytes [1—3].

An amphotericin B molecule features a lactone ring containing 7 conjugated double bonds and a chain of hydrophilic groups. The molecules has two ionizable groups — a carboxyl and a mycosamine ones [4].

In 1973 and 1974 Andreoli [5], Finkelstein and Holz [6] and De Kruijff and Demel [7] proposed a model of a sterol-amphotericin B channel. The authors assume that the channel consists of two half-pores formed on either side of the membrane. The cylindrical half-pore is formed by 8 antibiotic molecules interdigitated by 8 sterol molecules and spans half of the bylayer. In accordance with this model, the charged groups are located near the pore entrance. The outside of the half-pore is hydrophobic, the inside is hydrophilic due to the presence of the hydroxyl groups of the lacton ring. Recently, single ionic channels formed by amphotericin B and nystatin were observed and examined [8,9]. A channel is characterized by one conducting state and is subject to frequent changes from the conducting to a non-conducting state and vice versa.

The present work is aimed at studying the dependence of the channel properties, such as conductance, selectivity and the characteristic times, on the structure of individual parts of the molecule. Studies have been made of channels formed by amphotericin B derivatives with modified amino sugars and carboxyls, as well as by similar natural antibiotics, viz. nystatin and mycoheptin, with different structures of the lactone ring.

## Materials and Methods

Amphotericin B, nystatin and mycoheptin were purified in Leningrad Institute of antibiotics and enzymes. The extinction coefficient of amphotericin B, $E_{382} = 1500$.

Amphotericin B was chemically modified with respect to the polar amino and carboxyl groups. Antibiotics AM-1 and AM-2 are methylated derivatives of amphotericin B: AM-1 has a shielded positive charge on the nitrogen, antibiotic AM-2 has a shielded positive charge on the nitrogen and a neutralized negative charge at the carboxyl. We also used *N*-acetyl amphotericin B with a neutralized positive charge, and a methyl ester of amphotericin B with a neutralized negative charge.

Mycoheptin was initially obtained at the Leningrad Institute of Antibiotics, in 1967, by Konev and Tsyganov from *Streptoverticillium mycoheptinicum* [10].

The chemical structures of amphotericin B, nystatin and mycoheptin were determined [4,11,12]. The only difference in the molecules of these three antibiotics lies in the lactone ring structure. The polyene chain is the same in both amphotericin B and mycoheptin (heptaenes), while in the nystatin molecule one double bond is hydrogenated (tetraene). The hydrophilic chains of the lactone ring are different in all three antibiotics: nystatin and amphotericin B exhibit the same set of hydrophilic groups which, however, are differently arranged. Mycoheptin has another hydroxyl group substituted by a carbonyl. The stock solutions of the antibiotics were made up in dimethylsulfoxide and prepared just before each experiment. The antibiotics were introduced into both aqueous solutions at the same concentration, except for the cases where composite channels were examined.

Bilayer lipid membranes were formed from a solution of ox brain phospholipids (20 mg) and cholesterol (1 mg) in 1 ml of *n*-heptane, unless specified otherwise in what follows. The aqueous solutions normally contained 2 M KCl. The method is described in greater detail in an earlier paper [9].

## Results

*Effect of modification of the amino and carboxyl groups of the Amphotericin B molecule*

Fig. 1 represents the records of the current through the ionic channels formed by amphotericin B (line 1) and its derivatives (lines 2 through 5). Individual channels produced by these antibiotics differ only slightly in conductance. The selectivity of these channels is roughly the same: the zero current potentials by the KCl gradient (2 M—0.737 M) in the presence of amphotericin B and its derivatives are rather close and equal 18 ± 2 mV. The sign of the potential corresponds to a better permeability for $Cl^-$ than for $K^+$.

The channels formed by amphotericin B derivatives, as opposed to those formed by amphotericin B itself, remain conducting for a shorter period of time. As can be seen from the recordings in Fig. 1, the channels become nonconducting for some time, then are rendered conducting again. We assume that

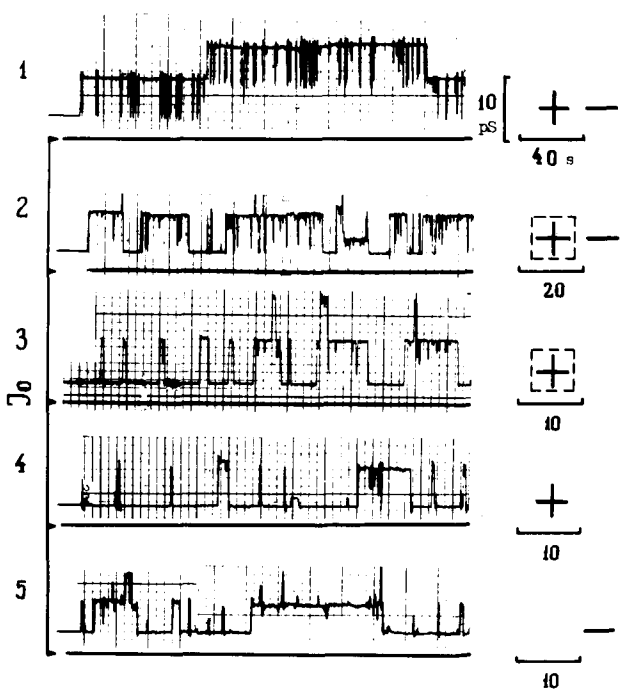


Fig. 1. Discrete variations in the membrane conductance in the presence of amphotericin B and its derivatives. Membrane potential —200 mV, 2 M KCl, pH 7.0, 23°C. Membrane solution, phospholipid : cholesterol = 20 : 1. 1, Amphotericin B $2 \cdot 10^{-8}$ M; 2, AM-1 $5 \cdot 10^{-8}$ M; 3, AM-2 $7 \cdot 10^{-8}$ M; 4, methyl ester of amphotericin B $1 \cdot 10^{-6}$ M; 5, *N*-acetyl amphotericin B $3 \cdot 10^{-7}$ M. The charges on the amino and carboxyl groups of the antibiotic molecules are shown on the right.

a new non-conducting state occurs, which shall be termed the 'inactive state' of a channel. In addition, two states persist, typical of an amphotericin B channel: an open state and a short-duration closed state. The mean lifetimes of a channel in the open and short-duration closed states are, respectively, $3 \pm 0.5$ and $0.13 \pm 0.05$ s [8]. These values hold for channels formed by derivatives. The combination of these two states shall be termed 'active state' of a channel.

Record 2 in Fig. 1 exhibits a change in the membrane current in the presence of antibiotic AM-1 in which the positive charge on the nitrogen is screened.

Comparison of records 1 and 2 in Fig. 1 indicates that the mean life-time of an active state ($T_A$) of the channel formed by AM-1 is much shorter than that of the amphotericin B channel ($6 \pm 1$ and 240 s, respectively). Statistical analysis shows the record 2 in Fig. 1 is representative of the behavior of a single channel.

In the presence of antibiotic AM-2, in which not only the positive charge at nitrogen is screened, but the carboxyl group charge is neutralized, the mean lifetime in the active state becomes still shorter: $1.6 \pm 0.16$ s (record 3 in Fig. 1). It is not clear whether one or several channels are functioning in this case. Therefore, determining the lifetime of a channel in the inactive state is impossible. Antibiotic AM-2 retains the positive charge on the nitrogen in the pH range from 3 to 11. In the whole of this pH range, the $T_A$ of the channels remains invariable.

In the case of a methyl ester of amphotericin B having only the positive charge similarly short lifetimes of channels in the active state are observed; just as in the case of AM-2 (record 4 in Fig. 1). A channel rarely remains in the active state for a longer period of time. The mean active state time is then equal to $2.5 \pm 0.25$ s.

*N*-Acetyl amphotericin B having only the negative charge produces a similar channel behavior. Longer active states of channels formed by this derivative are more common (record 5 in Fig. 1; $T_A = 3.0 \pm 0.3$ s). In acid media, *N*-acetyl amphotericin B loses the negative charge and becomes a neutral polyene. This results in a shorter $T_A$ (record 5 in Fig. 2). Under these conditions, potential-dependent behavior of channels is observed. This record shows that, as the membrane potential changes from 200 to 150 mV, the frequency at which channels change their states becomes twice as low.

*Effect of varying pH of aqueous solutions*

Another way to neutralize one of the charges of the amphotericin B molecule is to change pH of aqueous solutions beyond the p$K$ value of amino or carboxyl group. One could expect that this also would result in a shortening of $T_A$. Indeed at pH 8.0 $T_A$ becomes shorter than that at pH 4—7 (records 1,2 in Fig. 2). At pH 9.0 $T_A$ becomes still shorter and its value does not change if the pH of the solutions are made more basic. Record 3 shows current through a channel at pH 11.0. In acid solutions $T_A$ also decreases (record 4).

The conductance of an individual channel in the pH range of 3 to 11 remains the same. Cass et al. [13] have shown that the integral conductance of membranes in the presence of amphotericin B and nystatin is low at basic solutions. This effect of pH seems to be due to the shorter lifetime of channels in the active state.

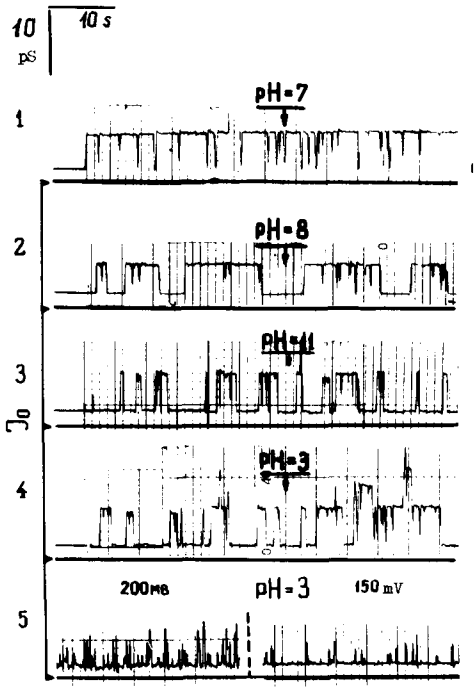


Fig. 2. Discrete variations in the membrane conductance in the presence of amphotericin B (1—4) (5) at different pH values and the membrane potential 200 mV. The conditions are the same as in Fig. 1. Given at the bottom (5) are the *N*-acetyl amphotericin B channel records at two values of the membrane potential.

*Effect of varying cholesterol concentration*

We have noticed that a higher cholesterol concentration in a membrane-forming solution increases the lifetime $T_A$ of a channel in the active state. We used both the usual lipid solution (phospholipid : cholesterol = 20 : 1) and a solution of 20 mg phospholipids and 10 mg cholesterol in 1 ml *n*-heptane (phospholipid : cholesterol = 20 : 10). At a phospholipid-to-cholesterol ratio of 20 : 1, the $T_A$ value of a channel formed by amphotericin B is 3 to 4 min, whereas at a ratio of 20 : 10, it exceeds 30 min (2 M KCl at pH 7.0). Under similar conditions, $T_A$ of a channel formed by amphotericin B derivatives with a single charged group (+ or —) increases too. Moreover, as the cholesterol concentration increases, $T_A$ becomes longer also in the case of antibiotics having both charges blocked (*N*-acetyl amphotericin B at pH 3.0 and methyl ester of amphotericin B at pH 11.0).

The lifetimes of the active state of channels formed by amphotericin B and its derivatives, depending on pH and the cholesterol concentration in the membrane, are listed in Table I.

*Dependence of channel properties on the lactone ring structure*

Shown in the right-hand portion of Fig. 3 is a record of the current through individual channels in 2 M KCl solutions. The amphotericin B channel features the maximum conductance (3.5 pS) and is subject to frequent changes from

the open to the closed state and vice versa. The nystatin channel has a lowe conductance (1.4 pS) and changes its states very seldom. The minimum cor ductance is exhibited by the mycoheptin channel (0.2 to 0.3 pS) with pract cally no changes from one state to the other.

Table I lists the zero current potentials at a tenfold ratio of the salt concer tration in two solutions, obtained on a membrane with a great number c channels ($10^3$ to $10^4$). The potential of 58 mV corresponds to an ideal anioni selectivity of channels. It can be seen that a membrane with amphotericin channels is more permeable to anions than to cations. The anionic selectivit of a membrane with mycoheptin is much poorer. $KNO_3$ even features a positiv potential, i.e. the nitrate anions pass through the channels worse than th cations. Mycoheptin and amphotericin B with similar polyene chains form ind vidual channels at concentrations of $2 \cdot 10^{-8}$ M (phospholipid : cholesterol 20 : 1). Nystatin having a broken double bond forms channels only at $10^{-7}$ M

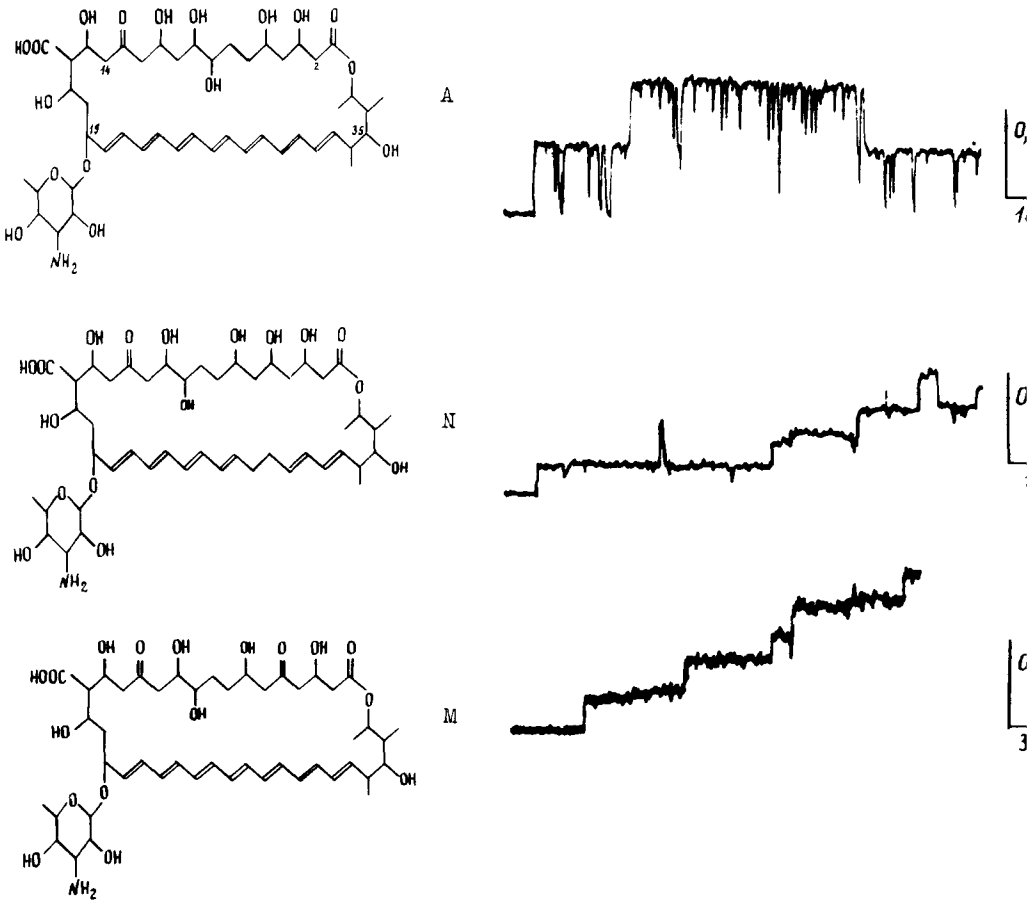


Fig. 3. On the left, structural formulae of amphotericin B (A), nystatin (N) and mycoheptine (M) mc cules. On the right, recors of the current through the membrane with these antibiotics. The conditic are same as in Fig. 1, except that the membrane potential is —100 mV. The antibiotic concentrations aqueous solutions are $2 \cdot 10^{-8}$ M (amphotericin B), $1 \cdot 10^{-7}$ M (nystatin), and $4 \cdot 10^{-8}$ M (mycohepti Note different current scales.

TABLE I

MEAN LIFETIME OF CHANNELS IN THE ACTIVE STATE ($T_A$) AND OPEN CHANNEL CONDUCTANCE AT VARIOUS CHOLESTEROL CONCENTRATIONS IN A MEMBRANE SOLUTION IN THE PRESENCE OF THE ANTIBIOTICS INVESTIGATED. ANTIBIOTIC CONCENTRATIONS NECESSARY TO FORM UNIT CHANNEL ARE GIVEN.

| Antibiotic | Cholesterol *n*-Heptane (mg/ml) | Conc. (M) | pH | Channel conductance pS ($V_m$ = 200 mV) | The mean lifetime of channels in active state ($T_A$, s) | Charges |
|---|---|---|---|---|---|---|
| Amphotericin B | 1 | $2 \cdot 10^{-8}$ | 7.0 | 6.5 | 260 ± 40 | + — |
| Amphotericin B | 1 | $2 \cdot 10^{-8}$ | 3.0 | 5 | 2.2 ± 0.4 | + |
| Amphotericin B | 1 | $7 \cdot 10^{-8}$ | 11.0 | 5 | 3.0 ± 0.6 | — |
| Am-1 | 1 | $5 \cdot 10^{-8}$ | 7.0 | 6.5 | 6.0 ± 1 | + — |
| Amphotericin B — methyl ester | 1 | $1 \cdot 10^{-6}$ | 7.0 | 6.5 | 2.5 ± 0.3 | + |
| Amphotericin B — methyl ester | 1 | $1 \cdot 10^{-5}$ | 11.0 | 6 | 1.8 ± 0.2 | |
| Amphotericin B — methyl ester | 10 | $1 \cdot 10^{-7}$ | 11.0 | 6 | 14 ± 4 | |
| N-acetyl amphotericin B | 1 | $3 \cdot 10^{-7}$ | 7.0 | 5 | 3.0 ± 0.3 | — |
| N-acetyl amphotericin B | 1 | $3 \cdot 10^{-7}$ | 3.0 | 6 | 0.3 ± 0.06 | |
| N-acetyl amphotericin B | 10 | $2 \cdot 10^{-8}$ | 3.0 | 6 | 9.2 ± 3 | |
| AM-2 | 1 | $7 \cdot 10^{-8}$ | 7.0 | 7 | 1.6 ± 0.2 | + |
| AM-2 | 2 | $4 \cdot 10^{-8}$ | 7.0 | 7 | 75 ± 20 | + |
| Nystatin | 1 | $1 \cdot 10^{-7}$ | 7.0 | 1.8 ± 0.2 | 220 ± 25 | + — |
| Nystatin | 1 | $5 \cdot 10^{-7}$ | 10.0 | 1.8 ± 0.2 | 7.3 ± 0.7 | — |
| Mycoheptin | 1 | $2 \cdot 10^{-8}$ | 7.0 | 0.25 ± 0.05 | — | + — |

Obviously, the rate of channel formation is determined by the polyene chain structure. This is corroborated by experiments with hydrogenated amphotericin B. This antibiotic without double bonds forms channels with an average conductivity of 4 pS and an active state lifetime of 2.7 ± 0.5 s. In this case, it takes a very high antibiotic concentration ($5 \cdot 10^{-7}$ M) to form individual channels, even at a high cholesterol concentration in the membrane (phospholipid : cholesterol = 20 : 10).

Antibiotics added on one side of the membrane do not increase conductance even at a concentration of $10^{-4}$ M (phospholipid : cholesterol = 20 : 1). Kasumov and Liberman [14] have shown that nystatin and amphotericin B added on different side of the membrane enhance its conductance as they interact with each other. It can be assumed that, in this case, composite nystatin-amphotericin channels are formed. Indeed if one aqueous solution contains $10^{-7}$ M of nystatin and the other, $2 \cdot 10^{-8}$ M of amphotericin B, composite channels are formed in the membrane. The conductance of these channels depends on the potential sign and the conductance value lies between those of 'pure' channels. For example, the conductance of a composite channel is 2.3 pS at +100 mV and 2.0 pS at —100 mV (the sign of the potential is given for a solution with nystatin). Composite channels are formed by any pair of the three antibiotics taken in concentrations required to obtain 'pure' channels. The conductance of an amphotericin B-mycoheptin pore is 1.4 pS at +100 mV and 0.6 pS at —100 mV (the sign of the potential is given for a solution with mycoheptin).

## Discussion

The above data indicate that the properties of a channel change to a considerable extent as the antibiotic molecular structure varies: (1) modification of the charged groups alters the ratio of life-times in the open and closed states and has practically no influence on the conductance of an open channel; (2) hydrogenation of double bonds in the polyene chain makes the formation of channels more difficult, and (3) variations in the hydrophilic chain of the lactone ring affect, primarily, the conductance and selectivity of a channel.

The presence of both negative and positive charges in an amphotericin B molecule provides for the channels remaining in the active state for a long period of time (240 s). It can be assumed that stability of the active state is associated with an electrostatic interaction between the amino group of one antibiotic molecule and the carboxyl group of an adjacent one. The loss of one of the polar group charges during chemical modification or while the pH value changes results in the lifetime of a channel in this state becoming much shorter (see Figs. 1, 2 and Table I). Screening of the positive charge of the amino group (AM-1) reduces $T_A$ 40 times. This can be accounted for by a weaker electrostatic interaction between the charges. Removal of the positive (*N*-acetyl amphotericin B) or negative charge (methyl ester of amphotericin B) renders $T_A$ still shorter. A similar effect is observed in the case of amphotericin B when one of the charges is removed by pH shift.

Removal of both charges results in still shorter active state durations ($T_A$ = 1.0 s for the methyl ester at pH 11.0, and 0.3 s for *N*-acetyl amphotericin at pH 3.0). Neutralization of the second charge possibly results in varying degree of interaction between the carboxyl group of the antibiotic molecule and the hydroxyl group of the cholesterol molecule. It was suggested earlier that the interaction between these groups plays an important role in stabilizing the complex [5,6,7].

A higher cholesterol concentration in the membrane always results in longer $T_A$ (Table I). To explain this let us assume that transition from the active to the inactive state is due to several cholesterol molecules leaving the complex. In this case, the complex becomes less stable, and the inactive state becomes more probable. As the cholesterol concentration in the membrane increases, the probability of cholesterol molecules returning into the complex becomes higher, hence, that of transition to the inactivate state becomes low.

As has been shown above, a hydrogenation of double bonds in the polyene chain of antibiotic causes channel formation to slow down. This seems to be due to the flexibility of the molecule at points where the double bonds break, which makes it more difficult for a complex with a cholesterol molecule to form. In fact, nystatin is more reluctant to enter into cholesterol-containing lipid monolayers than amphotericin B [15].

Comparison of the conductance and selectivity (Table II) of channels formed by three natural antibiotics suggests that rupture of a double bond in the polyene chain and rearrangement of groups in the polar chain of the lactone ring produce a less effect on the open channel properties than the appearance of another carbonyl group (instead of the hydroxyl group) in the polar chain. The appearance of this carbonyl group reduces the anionic selectivity and

TABLE II

ZERO CURRENT POTENTIALS REDUCED TO A TENFOLD RATIO OF ELECTROLYTE CONCENTRATIONS IN TWO SOLUTIONS. IN ALL CASES EXCEPT THOSE MARKED BY *, THE ELECTROLYTE CONCENTRATIONS IN THE TWO SOLUTIONS WERE 2 M AND 0.74 M, RESPECTIVELY. IN REDUCING THE POTENTIAL TO THE TENFOLD RATIO IT WAS ASSUMED THAT THE POTENTIAL LINEARLY DEPENDS ON THE LOGARITHM OF THE CONCENTRATION RATIO.

| Antibiotic | Salt | | | | | |
|---|---|---|---|---|---|---|
| | KF | KCl | KBr | $KNO_3$ | CsCl | LiCl |
| Amphotericin B | −57 | −42.5 | −43 | −21.5 | −39 | — |
| Nystatin | −46.5 | −47 * | −28.5 | +2 | −27 | −50 * |
| Mycoheptin | −44 | −21.5 | −16.5 | +43 | −30 | −25.5 |

* — data from Ref. 17 for electrolyte concentrations of 10 and 100 M.

causes a sharp (ten fold) decrease in the channel conductance. Suppose that the anion selectivity is due to a positive potential induced by OH dipoles inside the pore. A substitution of the OH group by a carbonyl one should result in a decrease of the positive potential and, consequently, in a lower anion-cation selectivity. It is more difficult to account for the sharp decrease in the channel conductance. In an amphotericin B molecule, there are already two carbonyl group. Addition of one more carbonyl in the mycoheptin molecule would hardly bring about a ten-fold decrease in conductance. We have shown that quarternary ammonium compounds 'plug' channels. A mycoheptin channel turned out to be close, in size, to the tetramethylammonium molecule, while an amphotericin B channel was found to be commensurate with the tetraethylammonium molecule [16]. So the lower channel conductance is more likely to be due to the smaller diameter of the mycoheptin channel.

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