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THE ACTION OF PIMARICIN, ETRUSCOMYCIN AND AMPHOTERICIN B ON LIPOSOMES WITH VARYING STEROL CONTENT

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Summary

1. The effect of pimaricin, etruscomycin and amphotericin B on the K^+ release from liposomes is strongly dependent on their sterol concentration.

Pimaricin and etruscomycin induce K^+ release from egg lecithin liposomes with cholesterol contents of more than 25 and 10 mol%, respectively, at polyene concentrations of 100 and 10 μg/ml, respectively. Amphotericin B shows a maximal effect at a cholesterol content of 20 mol% at a concentration of 0.4 μg/ml.

2. For liposomes containing ergosterol the sensitivity is shifted to a lower sterol content. All three polyenes show activity at 10 mol% ergosterol. The sensitivity for amphothericin B is increased approx. 15 times by the incorporation of ergosterol compared to cholesterol. The increase in sensitivity is much less for pimaricin and etruscomycin. The K^+ release is maximal at an ergosterol concentration of 30 mol%.

3. Pimaricin, etruscomycin and amphotericin B can induce K^+ release from erythrocytes without the release of haemoglobin at concentrations of 20, 2 and 1 μg/ml, respectively. For these polyenes a selective permeability change is also demonstrated for liposomes since K^+ is released but no [^{14}C]dextran. Filipin shows a nonselective release of solutes from erythrocytes and liposomes.

4. At cholesterol concentrations higher than 20 mol% and ergosterol concentrations higher than 10 mol%, etruscomycin, pimaricin and amphotericin B show little dependence of the bilayer thickness and are able to release K^+ from didocosenoyl phosphatidylcholine liposomes after addition of the polyene to one side of the membrane.

A possible mechanism is discussed.

Introduction

Polyene antibiotics are used for their fungocidal action. The presence of sterol in the membrane is a prerequisite for polyene antibiotic sensitivity. For various polyene antibiotics such as filipin, amphotericin B, nystatin, etruscomycin and pimaricin, several differences in effect have been described. Filipin is an uncharged polyene with the highest sterol affinity. Filipin-cholesterol complexes are visualized as pits with negative staining electron microscopy [1] and as particles (100—240 Å in diameter) in the hydrophobic core of the membrane, with freeze-fracture electron microscopy [2,3]. The filipin-sterol aggregates lead to a fragmentation of the membrane and cause release of all cytoplasmic components [4]. Amphotericin B and nystatin cause a selective change in permeability. These polyenes increased the permeability for small solutes such as K^+, Na^+, urea and erythritol in *Acholeplasma laidlawii* cells, containing 16 mol% cholesterol in the membrane, without the release of cytoplasmic enzymes such as glucose-6-phosphate dehydrogenase. Amphotericin B-cholesterol and nystatin-cholesterol complexes function as aqueous channels traversing the membrane [4,5]. For the amphotericin B action a detailed molecular mechanism is proposed [6,7]. Complexes of eight amphotericin B molecules and a similar number of sterol molecules are arranged so that a conducting pore is formed. An aqueous channel of 8 Å diameter is formed by the hydrophylic hydroxyl groups of the polyene antibiotic. The charged hydrophylic carboxyl group and mycosamine group are supposed to be at the membrane/water interface.

Little is known about the smaller polyene antibiotics etruscomycin and pimaricin. For estruscomycin a significant change in K^+ permeability in *A. laidlawii* cells could be demonstrated only at low temperatures (0°C) [4]. Primaricin was not able to produce permeability changes in *A. laidlawii* cells and egg lecithin liposomes, containing 16 mol% cholesterol [4]. These small polyene antibiotics (etruscomycin M_r 707 and pimaricin M_r 665) have however fungocidal activity; they do lyse erythrocytes and penetrate monolayers of cholesterol [8] and remove cholesterol from its interaction with phosphatidylcholine as determined by differential scanning calorimetry [16]. Freeze-fracture electron microscopy showed that pimaricin causes network particle aggregation in fungal membranes and particle aggregation in erythrocyte ghost [9]. It has been suggested that these polyenes might have a higher affinity for ergosterol than for cholesterol. It has been shown that the lipid composition and sterol content can affect the membrane sensitivity for some polyene antibiotics [17, 18]. In this paper the activity of amphotericin B, etruscomycin and pimaricin as a function of cholesterol and ergosterol concentration is studied, in order to achieve better understanding of the molecular mechanism especially of the smaller polyene antibiotics.

Experimental

Materials

Amphotericin B was supplied by the Squibb Institute for Medical Research, New Brunswick, NJ. Pimaricin (natamycin) by Gist Brocades, Delft. Etrusco-

mycin (lucensomycin) by Farmitalia, Milan, and filipin from the Upjohn Company Kalamazoo, MI. The polyene antibiotics were dissolved in Me_2SO (amphotericin B) or dimethyl formamide (etruscomycin and pimaricin) at a concentration of 1 mg/ml and were always freshly prepared. To dissolve pimaricin a drop of 0.1 N HCl was added. Egg lecithin and phosphatidic acid derived from it were obtained as described before [4]. 1,2-Didocosenoyl-*sn*-glycero-3-phosphatidylcholine was synthesized as describe elsewhere [10]. Cholesterol was obtained from Merck (Darmstadt), ergosterol from Sigma (St. Louis, MO). The compounds were pure as checked by thin-layer chromatography. [*carboxyl*-^{14}C]Dextran 1 μCi/mg in 250 mM sucrose 10 mM potassium phosphate buffer, pH 7.0, was obtained from New England Nuclear, Boston, MA.

K^+ and haemoglobin release from erythrocytes

Heparin-treated rabbit blood was centrifuged for 5 min at 3000 rev./min. The erythrocytes were washed three times with 155 mM NaCl and twice with 100 mM $CaCl_2$/10 mM Tris-HCl, pH 7.0. The polyene antibiotic dissolved in dimethyl formamide or Me_2SO was suspended in 10 ml 100 mM $CaCl_2$/10 mM Tris-HCl, pH 7.0, and then 50 μl of the erythrocyte suspension (haematocrit 50%) was added. After 10 min incubation the erythrocytes were centrifuged for 5 min at 3000 rev./min. Haemoglobin was determined in the supernatant at 540 nm. 100% haemoglobin release was determined by suspending the erythrocytes in distilled water. The K^+ release was determined with a K^+ sensitive electrode. 100% K^+ release was determined by addition of 50 μl 10% Triton X-100. The experiments were performed at 25°C.

K^+ and [^{14}C]dextran release from liposomes

Egg lecithin, or didocosenoyl phosphatidylcholine and cholesterol or ergosterol (total 40 μM) were mixed with phosphatidic acid (1.25 μM) and dispersed in 1 ml 150 mM KCl/10 mM Tris-HCl, pH 7.5 on a vortex mixer. The liposomes were dialyzed three times against 100 mM $CaCl_2$/10 mM Tris-HCl, pH 7.5, for 30 min. Egg lecithin was dialyzed at 0°C and didocosenoyl phosphatidylcholine at 22°C.

The K^+ efflux from the liposomes was measured with a K^+-specific electrode at 22°C [4]. The total release of K^+ was measured after the addition of 50 μl 10% Triton X-100. To measure the release of [^{14}C]dextran a mixture of 3.5 μM egg lecithin 1.5 μM ergosterol and 0.12 μM phosphatidic acid was dispersed in 25 μl [^{14}C]dextran in a buffer comprising 250 mM sucrose (0.25 μCi) and 75 μl 150 mM LiCl/10 mM Tris-HCl, pH 7.5. Liposomes were washed three times by centrifuging at 37 000 $\times$ *g* for 10 min and resuspending in the sucrose-LiCl buffer as above. LiCl was used for better sedimentation of the liposomes. To 4.8 ml buffer, 200 μl liposome suspension and 50 μl polyene antibiotic dissolved in dimethylformamide or dimethyl sulfoxide were added. After incubation the liposomes were centrifuged down and 0.8 ml samples of the supernatant were taken to determine the [^{14}C]dextran released. 10 ml counting medium was used (8 g 2,5-diphenyloxazol/0.2 g 2,2′-*p*-phenylen-bis(4-methyl-5-phenyl-oxazol)/1 l toluene/860 ml Triton X-100). The blank value was determined after the addition of 50 μl dimethyl formamide or Me_2SO. The total amount of [^{14}C]dextran entrapped in the liposomes was determined by the addition of 50 μl 10% Triton X-100.

Binding experiments

Liposomes were prepared as described above, except that the lipid was dispersed in 1 ml of 300 mM glucose/10 mM Tris-HCl, pH 7.5. To 10 ml of a 150 mM LiCl/10 mM Tris-HCl pH 7.5 solution, polyene antibiotic and 100 μl of a 40 mM liposome suspension were added. After an incubation time of 15 min, the liposomes were centrifuged for 15 min at 12 000 $\times g$. The pellet was dissolved in 2 ml $CHCl_3/CH_3OH$ (1 : 1, v/v) and the amount of polyene antibiotic was determined spectrophotometrically [11], amphotericin B at 408 nm, pimaricin and etruscomycin at 319 nm. All experiments were done at 25°C.

Results

To test whether the mechanism of pimaricin and etruscomycin action can be compared with that of filipin or of amphotericin B, the effects of these polyenes on erythrocytes were compared. Whether the polyene antibiotic-induced permeability changes are specific for small solutes is studied by measuring the K^+ and haemoglobin release from erythrocytes. For amphotericin B, the release of K^+ starts at a concentration of 0.3 μg/ml. At a concentration of 0.5 μg/ml approx. 50% of the K^+ is released, but no haemoglobin (Fig. 1A). Also, at longer incubation times no release of haemoglobin is observed (Fig. 1B). The selective permeability for small solutes introduced by amphotericin B is in agreement with earlier observations [4]. In the presence of filipin, however, at all concentrations at which there was release of K^+, haemoglobin was also released (Fig. 2). In the presence of etruscomycin and pimaricin there is a concentration range over which K^+ is released but little or no haemoglobin. At an etruscomycin concentration of 1.5 μg/ml and a pimaricin concentration of 20 μg/ml, approx. 50% of the K^+ is released from the erythrocyte but no haemoglobin (Figs. 3A and 4A). Even after an incubation time of 1 h there is still little or no release of haemoglobin, whereas 60% of the K^+ is released (Figs. 3B and 4B). Etruscomycin causes an increase of erythrocyte lysis of a few percent in 1 h. These results also show that, whereas etruscomycin and pimaricin are not able to release K^+ from *Acholeplasma laidlawii* cells and liposomes containing 16 mol% cholesterol, they are effective towards erythrocytes containing 50 mol% of cholesterol. To test whether the high sensitivity of the erythrocyte for these polyenes is due to membrane structure or to the high sterol content, liposomes were used with a varying concentration of cholesterol and ergosterol. The effect of the polyene antibiotics on the K^+ release and their binding to the liposomes was measured. Etruscomycin shows very little effect on the K^+ release of liposomes containing less than 20 mol% cholesterol. At higher cholesterol concentrations rapid K^+ release is observed, and this was maximal at 40 mol% cholesterol (Fig. 5A). In fungi, ergosterol is the main sterol. The K^+ release is increased by a factor of 2 when ergosterol is incorporated and is already maximal at 30 mol% ergosterol. The percent of etruscomycin binding is not significantly different for liposomes containing cholesterol or ergosterol. With pimaricin, release of K^+ is observed only at cholesterol concentrations of 40—50 mol% (Fig. 6A). A great enhancement of the K^+ release is found when ergosterol is incorporated at concentrations of 20—40 mol% (Fig. 6B). The rate is decreasing at concentrations of 50 mol%.

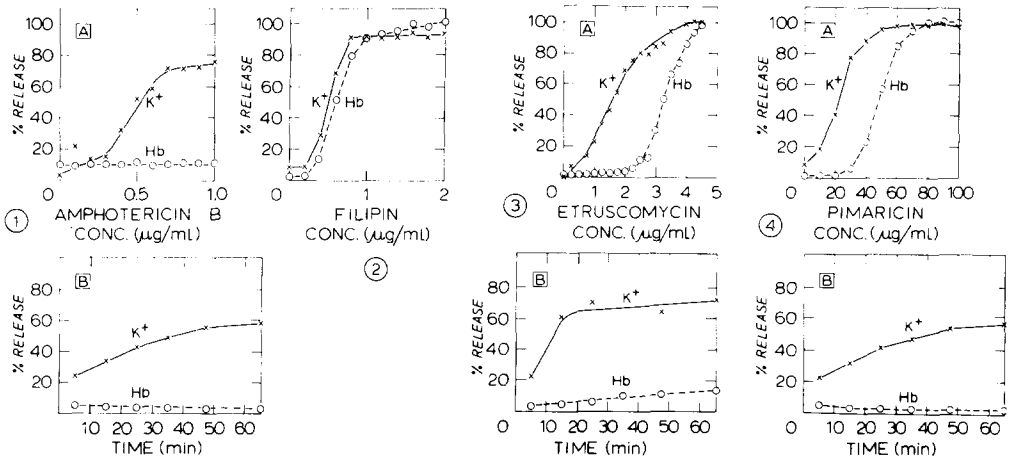

Fig. 1. A. Effect of amphotericin B on the K^+ and haemoglobin (Hb) release from rabbit erythrocytes, measured after 10 min. B. Effect of amphotericin B at a concentration of 0.5 μg/ml on the K^+ and haemoglobin release from rabbit erythrocytes.

Fig. 2. Effect of filipin on the K^+ and haemoglobin release from rabbit erythrocytes after 10 min.

Fig. 3. A. Effect of etruscomycin on the K^+ and haemoglobin release from rabbit erythrocytes after 10 min. B. Effect of etruscomycin at a concentration of 1.5 μg/ml on the K^+ and haemoglobin release from rabbit erythrocytes.

Fig. 4. A. Effect of pimaricin on the K^+ and haemoglobin release from rabbit erythrocytes, after 10 min. B. Effect of pimaricin at a concentration of 20 μg/ml on the K^+ and haemoglobin release from rabbit erythrocytes.

The binding of pimaricin to cholesterol- or ergosterol-containing liposomes is not very different except at 50 mol% sterol (Fig. 6A,B). A clear maximum in the K^+ release is demonstrated for amphotericin B at 20 mol% cholesterol. At lower, but also at higher, cholesterol concentrations, the K^+ release is less (Fig. 7A). Ergosterol gives an enormous stimulation of the amphotericin B activity. A 16-times lower amphotericin B concentration was used to measure the K^+ release from ergosterol-containing liposomes (Fig. 7B) than for measurement of the K^+ release from cholesterol-containing liposomes (Fig. 7A). Because of the spectrophotometric detectability of amphotericin B, the binding experiment were both done at a concentration of 0.4 μg/ml. The binding of ergosterol containing liposomes is higher than of cholesterol containing liposomes (Fig. 7A,B). In both cases a maximum in the binding is found, this being shifted to lower sterol concentrations in the case of ergosterol.

To prove that a selective permeability is induced in liposomes by etruscomycin and pimaricin as was found for amphotericin B, the release of [^{14}C]-dextran from liposomes was studied. (10 μg/ml) estruscomycin, (100 μg/ml) pimaricin and (0.05 μg/ml) amphotericin B showed no release of dextran from liposomes containing no or 30 mol% ergosterol.

The polyene antibiotic concentrations used gave maximal K^+ release from liposomes containing 30 mol% ergosterol (Figs. 5—7). 20 μg/ml filipin gave a complete release of dextran from liposomes containing 30 mol% ergosterol,

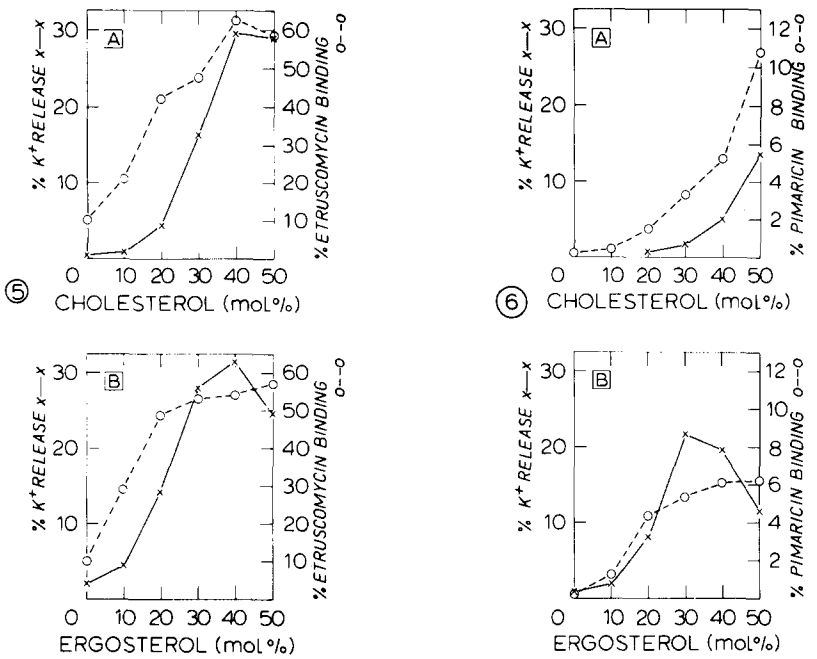

Fig. 5. A. Effect of cholesterol concentration in egg phosphatidylcholine liposomes on the K^+ release induced by etruscomycin at a concentration of 10 μg/ml measured after 3 min and the etruscomycin binding to the liposomes measured after 15 min. B. Effect of ergosterol concentration in egg phosphatidylcholine liposomes on the K^+ release induced by etruscomycin at a concentration of 10 μg/ml measured after 3 min and the etruscomycin binding to the liposomes measured after 15 min.

Fig. 6. A. Effect of cholesterol concentration in egg phosphatidylcholine liposomes on the K^+ release induced by pimaricin at a concentration of 100 μg/ml measured after 3 min and the pimaricin binding to the liposomes measured after 15 min. B. Effect of ergosterol concentration in egg phosphatidylcholine liposomes on the K^+ release induced by pimaricin at a concentration of 100 μg/ml measured after 3 min and the pimaricin binding to the liposomes measured after 15 min.

but also in the absence of sterol 44.8% dextran is released. Additionally, in previous studies it was shown that filipin induces significant release of K^+ from sterol-free liposomes [4].

It has been shown before that amphotericin B is unable to cause a change in the K^+ permeability of liposomes of didocosenoyl phosphatidylcholine containing 16 mol% cholesterol unless the polyene antibiotic is added to both sides of the membrane. It is thought that the length of the half pore formed by amphotericin B is insufficient to span the bilayer of this phospholipid [13]. To see whether the bilayer thickness is also a limiting factor for the action of the smaller polyene antibiotics, the effect of a long-chain phosphatidylcholine and sterol concentrations on the release of K^+ was studied (Fig. 8). The one-sided addition of amphotericin B to didocosenoyl phosphatidylcholine liposomes containing less than 20 mol% cholesterol had no effect. K^+ although the amphotericin B concentration was 50-times higher than in the experiments with egg lecithin-cholesterol liposomes. Also, etruscomycin and pimaricin were able to introduce K^+ release from didocosenoyl phosphatidylcholine liposomes containing more than 20 mol% cholesterol.

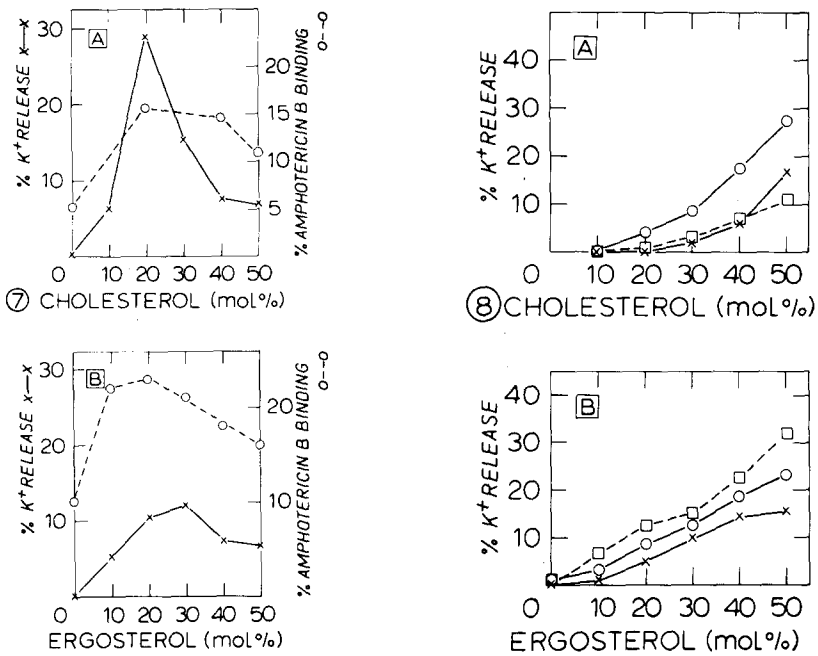

Fig. 7. A. Effect of cholesterol concentration in egg lecithin liposomes on the K^+ release induced by amphotericin B at a concentration of 0.4 μg/ml measured after 3 min and the amphotericin B binding to the liposomes measured after 15 min. B. Effect of ergosterol concentration in egg phosphatidylcholine liposomes, induced by amphotericin B at a concentration of 0.025 μg/ml measured after 3 min and the amphotericin B binding at a concentration of 0.4 μg/ml to the liposomes measured after 15 min.

Fig. 8. A. Effect of cholesterol concentration in didocosenoylphosphatidylcholine liposomes on the K^+ release induced by pimaricin (100 μg/ml) (X); amphotericin B (20 μg/ml) (□); etruscomycin (10 μg/ml) (○), measured after 3 min. B. Effect of ergosterol concentration in didocosenoylphosphatidylcholine liposomes on the K^+ release induced by pimaricin (100 μg/ml) (X); etruscomycin (10 μg/ml) (○); amphotericin B (0.5 μg/ml) (□).

The incorporation of ergosterol in liposomes of didocosenoyl phosphatidylcholine gave stimulation of the K^+ release at lower sterol concentrations. For amphotericin B a 20-times higher concentration was used than in experiments with egg lecithin-ergosterol liposomes. For etruscomycin and pimaricin the same concentration was used as with egg lecithin liposomes.

Discussion

Erythrocytes are sensitive towards polyene antibiotics, even towards the weaker ones such as pimaricin [14]. The high sensitivity is thought to be primarily due to the high cholesterol content of the erythrocyte membrane. With respect to the mechanism of polyene antibiotic action, it is of interest to know whether pimaricin and etruscomycin can induce selective permeability towards small solutes. In the case of pimaricin, etruscomycin and amphotericin B a distinct concentration range is found over which K^+ is released but not haemoglobin. In the case of filipin there is a simultaneous release of K^+ and haemoglobin at all concentrations. A 50% K^+ release after 10 min is found for

pimaricin, etruscomycin, amphotericin B and filipin at concentrations of 22, 1.7, 0.55 and 0.50 μg/ml, respectively. This order is in agreement with the literature [12]. Earlier experiments with liposomes of egg lecithin containing 15.7 mol% cholesterol showed that pimaricin in concentrations of 100 μg/ml was unable to induce K^+ release. For etruscomycin a K^+ release of 2% was found at a concentration of 50 μg/ml [4]. Pimaricin was also unable to induce K^+ release in *Acholeplasma laidlawii* cells containing 16 mol% cholesterol. Etruscomycin had little effect on these cells at a concentration of 20 μg/ml at 25°C but did show an effect at 0°C [4]. This study clearly demonstrates that the sterol concentration of the membrane is of critical importance for the activity of the polyene antibiotics. In agreement with the above experiments, the results in Figs. 5 and 6 show that at 16 mol% cholesterol there is still no effect of pimaricin and only very little effect of etruscomycin on the K^+ release. However, at higher cholesterol concentrations membranes become sensitive towards these polyene antibiotics. This probably also explains the erythrocyte sensitivity for etruscomycin and pimaricin. In erythrocytes and liposomes the smaller amphiphatic polyenes induce specific permeability changes, possibly by the formation of pores like the larger amphiphatic polyenes.

The polyene-sterol interaction is strongly dependent on the chemical structure of the sterol molecule [4]. This is also apparent for the K^+ release induced by the smaller amphiphatic polyene antibiotics. Ergosterol significantly increases the sensitivity for etruscomycin and pimaricin, especially at lower ergosterol concentrations. At a concentration of 16 mol% ergosterol a significant release of K^+ is demonstrated at concentrations of 10 and 100 μg/ml for etruscomycin and pimaricin, respectively. This could explain the sensitivity of fungi for these antibiotics. The protoplast membrane lipids from *Saccharomyces cerevisiae* contain 20—30 mol% ergosterol [15]. At an ergosterol content of 30 mol%, egg lecithin liposomes show a maximal effect of pimaricin, etruscomycin and amphotericin B (Figs. 5—7).

It is remarkable that there is a maximum in the sterol concentration of liposomes for the polyene antibiotic sensitivity. For etruscomycin and pimaricin this is especially clear with ergosterol, since the sensitivity is at lower sterol concentrations for ergosterol than for cholesterol (Figs. 5 and 6). For amphotericin B there is a maximum with cholesterol and ergosterol (Fig. 7). It has been shown previously that in liposomes containing 15.7 mol% cholesterol, a one-sided addition of amphotericin B is sufficient for egg lecithin, whereas for didocosenoyl phosphatidylcholine a two-sided addition of this polyene antibiotic is required [13]. In this paper it is shown that at cholesterol concentrations higher than 20 mol% and with ergosterol also at lower concentrations, one-sided additions of pimaricin, etruscomycin and amphotericin B are able to induce K^+ release from didocosenoyl phosphatidylcholine liposomes. These results can possibly be rationalised with the refined model for the amphotericin B action on membranes as proposed by van Hoogevest and de Kruijff [13]. They assume that half pores formed by the polyene antibiotic-sterol complex in the outer monolayer might have mobile carrier characteristics and shuttle up and down in the bilayer. A similar mechanism can be proposed for the small amphiphatic polyene antibiotics, etruscomycin and pimaricin. At low sterol concentrations polyene antibiotic-sterol complexes will be formed, as evi-

denced by the binding experiments, but no or only very few intact pores can be formed because of the lower sterol affinity of these polyene antibiotics. Alternatively, as in the case of didocosenoyl phosphatidylcholine, the efficiency of the pore might be smaller. At higher sterol concentrations more polyene antibiotic binding occurs and more pores will be formed, which results in increased permeability changes. At the same time however membrane viscosity will increase, at higher sterol concentrations, resulting in a decreased shuttling rate of the pore. This could lead to the observed optimal sterol concentration for the K^+ release.

Kitajima et al. [9] did not find changes in whole red blood cells as detected by negative staining and freeze-fracture electron microscopy, after treatment with pimaricin, nystatin and amphotericin B, but did find alterations on fungal plasma membranes. The present study shows, however, that etruscomycin, pimaricin and amphotericin B can increase the membrane permeability of intact erythrocytes as well as of liposomes containing cholesterol or ergosterol. The models for the polyene antibiotic-sterol complex proposed by Kitajima et al. [9] do not account for the selective permeability changes described in this paper.

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