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POLYENE ANTIBIOTIC–STEROL INTERACTIONS IN MEMBRANES OF *ACHOLEPLASMA LAIDLAWII* CELLS AND LECITHIN LIPOSOMES

I. SPECIFICITY OF THE MEMBRANE PERMEABILITY CHANGES INDUCED BY THE POLYENE ANTIBIOTICS

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SUMMARY

1. The effect of filipin, amphotericin B, nystatin, etruscomycin and pimaricin upon the permeability properties of *Acholeplasma laidlawii* cells and egg lecithin liposomes was investigated. When cholesterol was present in the membrane the different polyene antibiotics produced permeability changes which were different for the various antibiotics.

2. Filipin disrupted the membrane structure, after the interaction with cholesterol, so that both small ions such as K^+ and large protein molecules like glucose-6-phosphate dehydrogenase are released.

3. Amphotericin B, nystatin and etruscomycin produced specific permeability changes which indicate that these antibiotics create aqueous pores of specific size (about 8 Å in diameter) in the membrane after the interaction with cholesterol.

4. Pimaricin was not able to produce permeability changes in *A. laidlawii* cells and egg lecithin liposomes.

5. Various sterols were incorporated in *A. laidlawii* and liposomal membranes after which the interaction of filipin and amphotericin B with these membranes was investigated by ultraviolet spectroscopy and K^+ permeability. Only those sterols which had a 3β-OH group, a planar molecule and hydrophobic side chain at C_{17} were able to interact with these polyene antibiotics and thereby enhance the membrane permeability.

INTRODUCTION

Polyene antibiotics can interact with and cause permeability changes in a variety of model and biological membranes. The presence of sterols in the membrane is a requirement for polyene antibiotic sensitivity. Several differences in the effect of the various polyene antibiotics upon the membrane permeability are described in the literature (see reviews of Kinsky [1, 2]). Amphotericin B and nystatin are thought

to form aqueous pores of 4–7 Å [3] or 7–10 Å [4] in diameter in cholesterol-containing black lipid membranes, whereas the filipin–cholesterol complexes visualised as pits with negative staining electron microscopy were interpreted as aqueous channels which would permit large molecules to pass through the membranes [1, 5]. However, recent freeze-etch electron microscopy studies demonstrated that these pits cannot represent aqueous pores but must be interpreted as filipin–cholesterol complexes present in the hydrophobic core of the membrane [6, 7]. Important unresolved questions which remain are: (1) Do amphotericin B and nystatin create pores in the liposomal model membrane and the biological membrane? (2) How can we interpret the filipin-induced permeability changes?

In this paper we can compare systematically the permeability altering properties of filipin, amphotericin B, nystatin, etruscomycin and pimaricin in membranes of egg lecithin-sterol liposomes and *Acholeplasma laidlawii* cells. This organism is very suitable for such studies because depending on the presence of sterols in the growth medium membranes can be obtained which contain no or up to 10% (w/w of total lipids) sterol [8, 9].

MATERIALS

Polyene antibiotics and sterols used in this study are from the same sources as described in previous publications [8, 12, 13]. Stock solutions of antibiotics except amphotericin B were prepared daily by dissolving 1–10 mg in 1.0 ml dimethyl formamide. Amphotericin B was dissolved in dimethylsulfoxide. Egg lecithin was isolated and purified as described by Pangborn [10]. Phosphatidic acid was obtained from egg lecithin by degradation with a crude phospholipase D preparation obtained from savoy cabbage.

A. laidlawii cells

A. laidlawii strain B cells were grown in media supplemented with various fatty acids and sterols as described before [8]. Cells in the logarithmic phase were washed once and suspended in 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) at a concentration of 3 mg cell protein per ml. In some experiments the cells were washed and suspended in 150 mM NaCl–10 mM Tris–HCl buffer (pH 7.5).

Preparation of liposomes

(*a*) *Liposomes for K^+ leak experiments.* Liposomes were prepared by dispersing 40 μmoles egg lecithin and 1.6 μmoles phosphatidic acid with or without 7.75 μmoles sterol (15.7 mole%) in 1.0 ml 150 mM KCl at room temperature. The lipid dispersion was sonicated for 1 min under N_2 at maximum power with a Branson sonifier. The liposomes were dialysed 3 times against 200 ml of 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) at 0 °C.

(*b*) *Liposomes for turbidity measurements.* As (*a*) except that 150 mM KCl, 300 mM glucose or 300 mM erythritol each buffered with 10 mM Tris–HCl buffer (pH 7.5) was trapped in the liposomes. The dialysis step was omitted.

(*c*) *Liposomes for glucose leak experiments.* As (*a*) except that 100 mM KCl + 100 mM glucose +50 mM Tris–HCl buffer (pH 8.0) was trapped in the liposomes.

The liposomes were dialysed at 0 °C 2 times against 150 mM NaCl–50 mM Tris–HCl buffer (pH 8.0) and 2 times against 100 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 8.0).

Spectrophotometric measurements

All spectrophotometric measurements were performed on a Perkin–Elmer two wavelength, double-beam spectrophotometer Model 356, operating in the split-beam mode using thermostated 1.0-cm cuvettes. The system in which the effect of *A. laidlawii* cells and egg lecithin liposomes with varying sterol composition upon the ultraviolet spectra of filipin and amphotericin was measured consisted of 1.0 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) and 100 µl cells (approx. 300 µg cell protein) or 50 µl liposomes (2 µmoles egg phosphatidylcholine). To this solution 20 µg filipin or amphotericin B was added. The mixture was agitated on a Vortex mixer for 30 s. The solution was transferred to the measuring cuvette and the spectra of the antibiotics were recorded after 5 min [12, 13]. Appropriate base line corrections were made.

Measurement of release of K^+ from A. laidlawii cells and liposomes

To 5 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5), 100–500 µl *A. laidlawii* cells or 50 µl liposomes were added. The loss of K^+ after the addition of the polyene antibiotics was measured using a potassium specific glass electrode [14]. The rate and extent of the polyene antibiotic induced K^+ release from liposomes and cholesterol containing *A. laidlawii* cells grown on different days showed variations up to 30–40%. Cultures inoculated from one stock culture and isolated on the same day showed variations of less than 10%. For this reason comparative studies were always done on cells inoculated from one stock culture and harvested at the same time.

Measurements of glucose release from liposomes

The release of glucose from liposomes was followed spectrophotometrically at 339 nm in a modification of the system described by Demel et al. [15]. The measuring cuvette contained 1.0 ml 100 mM $MgCl_2$–50 mM Tris–HCl buffer (pH 8.0), 10 µl hexokinase (6.7 mg protein/ml), 10 µl glucose-6-phosphate dehydrogenase (3.4 mg protein/ml), 50 µl 0.01 M $NADP^+$ and 50 µl ATP (12 mg/ml). The reference cuvette containing 1.12 ml $MgCl_2$ buffer. To both cuvettes 10 µl of liposomes was added. After all the glucose outside the liposome was converted via $NADP^+$ to 6-*P*-gluconо-δ-lactone in both cuvettes, polyene antibiotic was added. This is done because the polyenes have high absorbances at 339 nm which can change after the interaction with cholesterol containing membranes [12, 13].

Measurement of glucose-6-phosphate dehydrogenase release from A. laidlawii cells

The release of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase [16] from cells was recorded continuously by measuring the rate of NADPH formation at 339 nm. Both the reference and the measuring cuvette contained 1.0 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5), 0.261 µmole $NADP^+$ and 100 µl cell suspension. In addition the measuring cuvette contained 0.5 µmole glucose 6-phosphate. 20 µg of antibiotic was added at the same time to both cuvettes. The lysis of the cells was also measured by determining the glucose-6-phosphate dehydrogenase activity in the supernatant of a cell suspension at time intervals after the addition of the antibiotic.

RESULTS

Polyene antibiotic induced K^+ release from A. laidlawii cells and egg lecithin liposomes

Low concentrations of filipin, amphotericin B, nystatin and etruscomycin induce a strong K^+ leak from cholesterol grown *A. laidlawii* cells (Fig. 2). At these same concentrations, no K^+ leak is observed from cells grown in the absence of cholesterol.

Fig. 2 shows that filipin, amphotericin B and nystatin induce a leak of K^+ from egg lecithin liposomes which is significantly enhanced by the incorporation of 15.7 moles% cholesterol. This is in agreement with the studies of Kinsky et al. [19]. With etruscomycin at 25 °C (50 μg/ml) we observed only about 2% K^+ loss from the cholesterol containing egg lecithin liposomes. With pimaricin up to 100 μg/ml, we observed no K^+ loss from 15.7 and 50 mole% cholesterol egg lecithin liposomes nor from cholesterol grown *A. laidlawii* cells. The order of potency of inducing the K^+ leak from cholesterol containing liposomes and *A. laidlawii* cells is amphotericin B >

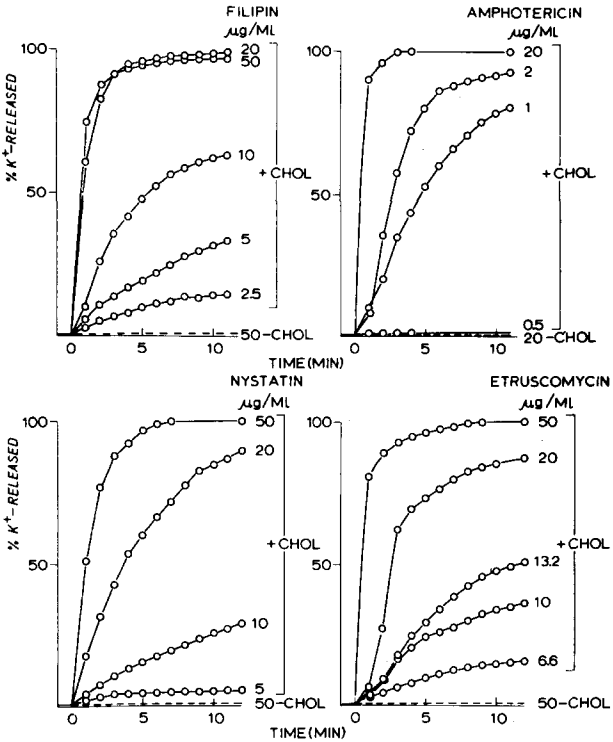

Fig. 1. Effect of various concentrations of filipin, amphotericin B, nystatin and etruscomycin upon the K^+ release from *A. laidlawii* cells grown without or with 25 mg cholesterol per l of culture. Test cuvette contained 5 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) and 100 μl cell suspension (300 μg cell protein). Filipin, amphotericin B and nystatin were tested at 25 °C, etruscomycin at 0 °C. Etruscomycin at 25 °C induces almost no K^+ leak from the cells (cf. [17] and Fig. 5). K^+ leak from cells grown without cholesterol was identical for all concentrations antibiotic tested. All leaks are corrected for the slow passive efflux of K^+ from the cells. The polyene antibiotics were added at time zero up to the concentrations indicated in the figure.

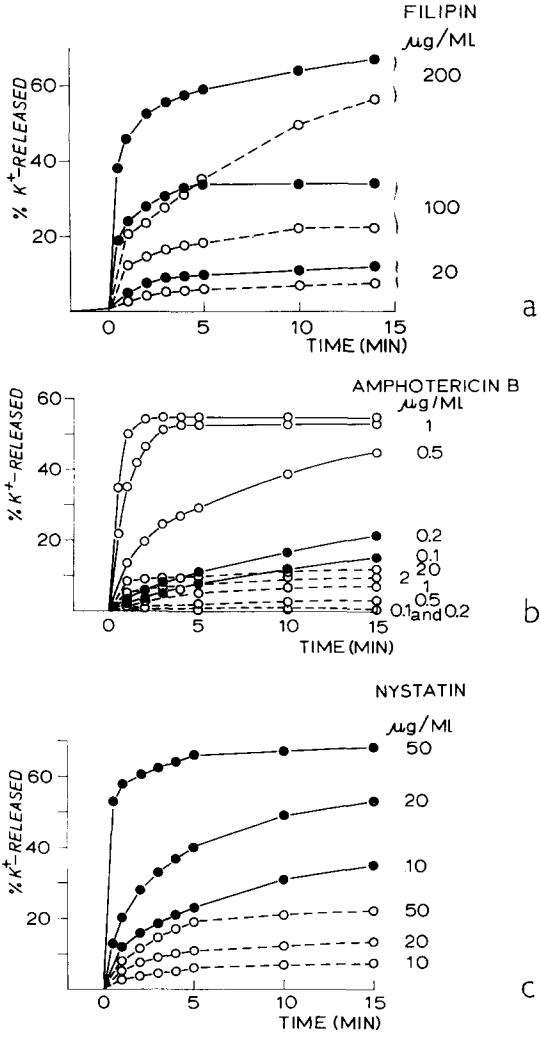

Fig. 2. Effect of various concentrations of filipin (a), amphotericin B (b) and nystatin (c) upon the K^+ release at 25 °C from liposomes prepared from egg lecithin with or without 15.7 mole % cholesterol. Test system as described in Materials and Methods. All leaks corrected for the slow passive efflux of K^+ from the liposomes. ●–●, with cholesterol; ○- - -○, without cholesterol.

nystatin > filipin > etruscomycin > pimaricin (Figs 1–3). In addition to the relatively high polyene antibiotic induced K^+ leak from the sterol-free liposomes, another important difference in the effect of the polyenes upon the liposomes and *A. laidlawii* cells is incomplete release of K^+ from the liposomes even at high concentrations of antibiotic. We suggest that this is caused by the multilamellar system of the liposomes because the extent of amphotericin B and filipin induced K^+ loss was increased up to 90% of the total amount of trapped K^+ when the liposomes were highly sonicated. Apparently only the cholesterol in the outermost layer of the liposomes is available for the antibiotics.

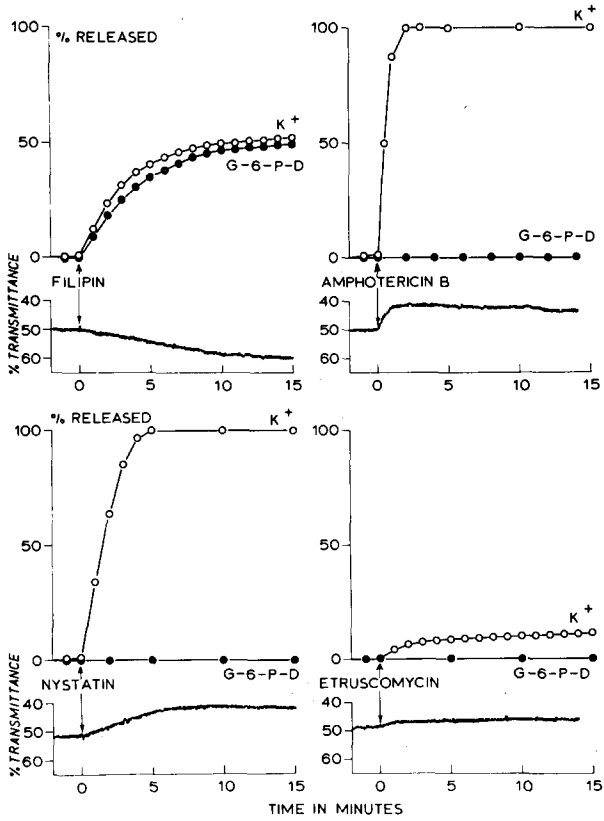

Fig. 3. Effect of filipin, amphotericin B, nystatin and etruscomycin upon the K^+ and glucose-6-phosphate dehydrogenase (G-6-P-D) release from and the turbidity of a cell suspension of cholesterol grown *A. laidlawii* cells. All measurements were done at 25 °C in the system described in the legend of Fig. 1 and Materials and Methods. The change in turbidity was measured at 542 nm. The polyene antibiotics were added at time zero up to a concentration of 20 μg/ml.

In order to investigate whether the polyene antibiotic induced K^+ leak from *A. laidlawii* cells is a result of a disruption of the membrane or of a more specific permeability change we examined the extracellular fluid for larger molecules like the cytoplasmic enzyme glucose-6-phosphate dehydrogenase. Fig. 3 shows the filipin, amphotericin B, nystatin and etruscomycin induced release of K^+ and glucose-6-phosphate dehydrogenase from, and the turbidity of, suspensions of cholesterol grown *A. laidlawii* cells.

It is obvious that only in the case of filipin is the K^+ loss accompanied by a release of glucose-6-phosphate dehydrogenase. Apparently the cells are lysed, as reflected by the increase in transmittance of the cell suspension. Under the phase contrast microscope only membrane fragments are visible. Thin sections performed by Drs A. J. Verkleij in our laboratory, also showed extensive destruction of the cells (Fig. 4). The above experiments were carried out in isotonic $CaCl_2$ buffer (pH 7.5). In isotonic NaCl buffer at pH 7.5 the filipin induced release of glucose-6-phosphate dehydrogenase from the cells proceeds at the same rate as in $CaCl_2$; however, the

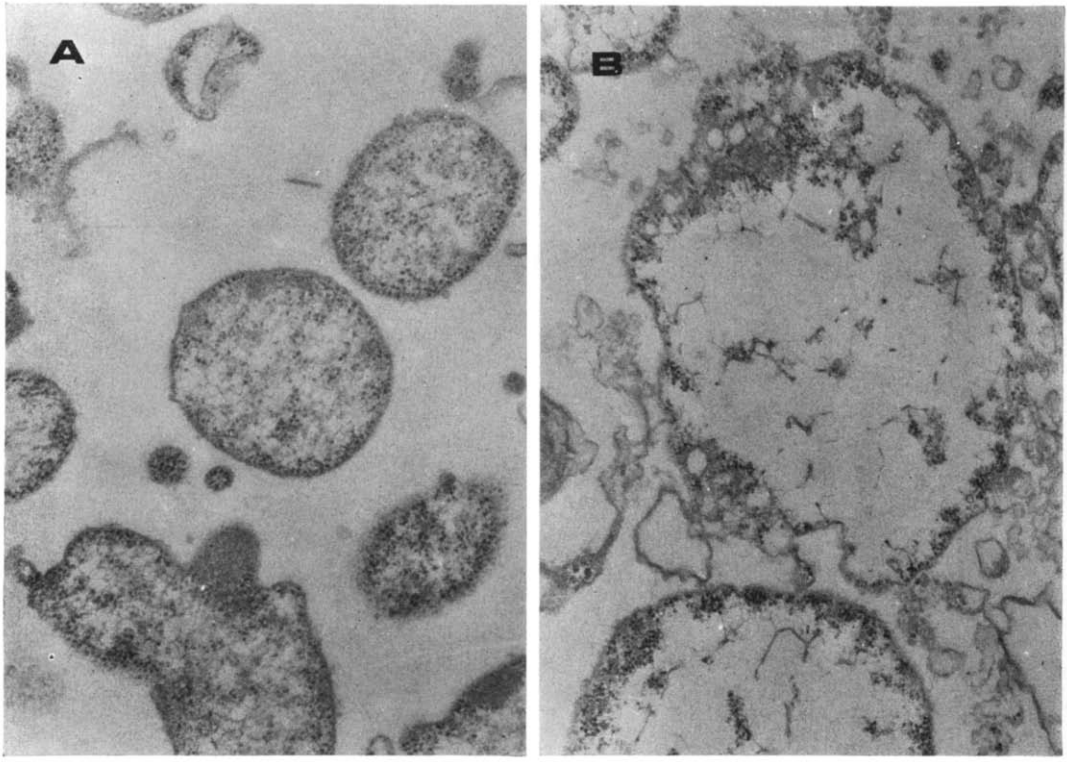

Fig. 4. Effect of filipin upon the morphology of the *A. laidlawii* cell. A. Micrograph of cholesterol grown *A. laidlawii* cells (60 μg cell protein/ml) in 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5), incubated for 10 min at 25 °C without filipin. B. Micrograph of the same cell suspension incubated for 10 min at 25 °C in the presence of filipin (20 μg/ml). After the incubation the cells were spun down at 0 °C. The pellet was suspended in isotonic veronal–acetate buffer (pH 8.0). Samples were fixed with OsO_4 and stained with uranyl acetate and lead citrate. Final magnification 40 000×.

increase in transmittance of the cell suspension is much larger. In fact, the solution becomes nearly clear, and even after prolonged high speed centrifugation no membrane pellet can be recovered. Apparently, the Ca^{2+} aggregates the membrane fragments formed during filipin action.

With amphotericin B, nystatin and etruscomycin at 0 and 25 °C no increase in glucose-6-phosphate dehydrogenase activity was observed, whereas a strong K^+ release is induced (Fig. 3). Apparently the membrane remains impermeable to glucose-6-phosphate dehydrogenase, glucose 6-phosphate and $NADP^+$ after the action of these antibiotics. Under the phase contrast microscope and in thin sections the cells appear to be intact although somewhat shrunken. This shrinkage of the cells in isotonic $CaCl_2$ is confirmed by an increase in turbidity following addition of these antibiotics (Fig. 3). Because K^+ is lost from the cells we conclude that Ca^{2+} does not enter the cell or enters it very slowly. Kinsky [21] observed a similar phenomenon with Neurospora protoplasts. Filipin produces a decrease in absorbance of a protoplast suspension in isotonic sucrose, which was attributed to cell lysis. Nystatin and amphotericin B only produced an increase in absorbance, which is explained by the authors as indicating a shrinking of the cells.

Specificity of the amphotericin B and nystatin induced permeability changes

In order to define the amphotericin B and nystatin induced permeability changes in the cholesterol-containing *A. lailawii* cell membrane more specifically, we investigated the change in turbidity (indicating swelling or shrinking) of the cells suspended in different isotonic media after the addition of these antibiotics (Fig. 5). In isotonic solutions of the chlorides of Ca^{2+}, Mg^{2+}, Li^+, Na^+, and Rb^+ we observed a shrinking after amphotericin B and nystatin addition only in $CaCl_2$ and $MgCl_2$. Apparently Ca^{2+} and Mg^{2+} cannot pass through the antibiotic modified membrane. With all monovalent cations, we observed cellular swelling (Fig. 5) which can be explained by an amphotericin B and nystatin induced increased permeability of these ions through the membrane. The rate of swelling is the highest in RbCl and decreases in the order $Rb^+ > K^+ > Na^+ > Li^+$, which is the order of the size of the hydrated ions. With nystatin we observe a stronger dependence on the size of the ion than with amphotericin B. Fig. 5 further shows the turbidity changes of cells in different isotonic nonelectrolyte solutions. Sucrose and glucose, in contrast to urea and erythritol cannot pass the amphotericin B modified membrane. In solutions of the 5 carbon sugars, ribose and xylose, the cells first shrink and then swell, presumably because the efflux of K^+ from the cells in the first 2 min after amphotericin B addition is greater than influx of these sugars. When the concentration gradient of K^+ has disappeared, the

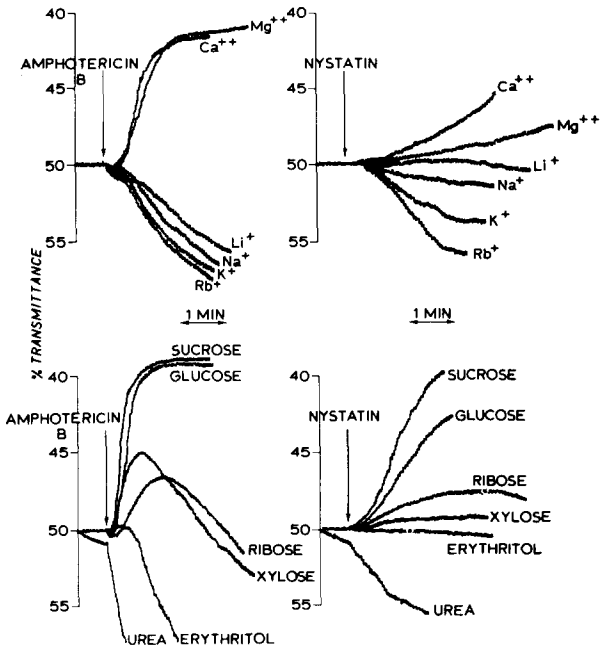

Fig. 5. Effect of amphotericin B and nystatin upon the turbidity of cholesterol grown *A. laidlawii* cells suspended in various isotonic media. Measurements were carried out at 0 °C in order to decrease the passive flux of urea and erythritol. The cells were once washed in 150 mM NaCl+10 mM Tris–HCl buffer (pH 7.5). The cuvette contained 5 ml of the desired solution, (300 mosM buffered with 10 mM Tris–HCl buffer (pH 7.5)), 100 μl cells (300 μg cell protein) was added. After about 2 min the antibiotic was added (arrow, final concn 20 μg/ml). The tracings are corrected for the small decrease in transmittance caused by amphotericin B.

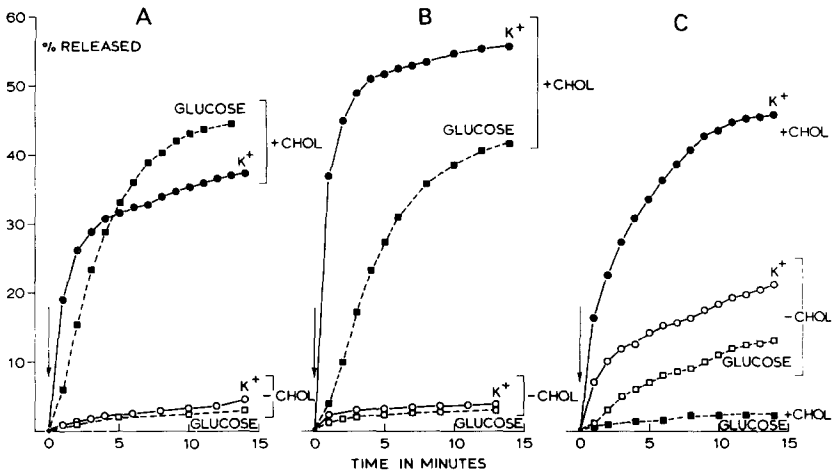

Fig. 6. Effect of filipin, amphotericin B and nystatin upon the K^+ and glucose release at 25 °C from liposomes prepared from egg lecithin with or without 15.7 mole % cholesterol. 100 mM KCl—100 mM glucose+50 mM Tris–HCl buffer (pH 8.0) was trapped in the liposomes. Procedures as described in Materials and Methods. Final polyene antibiotic concentrations: (A) filipin 20 μg/ml; (B) amphotericin B 1 μg/ml; (C) nystatin 20 μg/ml.

relatively slow influx of these sugars caused swelling. From this we conclude that molecules smaller than glucose and erythritol can pass the amphotericin B, respectively, nystatin-modified *A. laidlawii* cell membrane. For etruscomycin we observed a similar swelling-shrinking pattern of the cells as for amphotericin B and nystatin (data not shown).

The liposome system is more convenient to study the permeability characteristics of the amphotericin B and nystatin modified membrane. Various markers can be trapped and their release can be followed directly in the appropriate test system, or

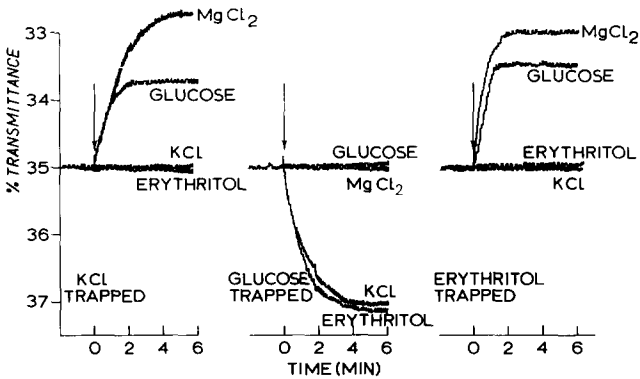

Fig. 7. Effect of amphotericin B upon the turbidity of egg lecithin–cholesterol (15.7 mole %) liposomes at 0 °C. 150 mM KCl, 300 mM glucose or 300 mM erythritol, each solution buffered with 10 mM Tris–HCl buffer (pH 7.5), was trapped in the liposomes. 20 μl liposomes was suspended in 5 ml 300 mM glucose, 100 mM $MgCl_2$, 150 mM KCl or 300 mM erythritol (buffered with 10 mM Tris–HCl buffer (pH 7.5)). After about 2 min amphotericin B was added (arrow) such that the final concentration was 8 μg/ml.

indirectly by following the turbidity of the liposomes when they are suspended in different isotonic media. In Fig. 6 are shown the results of an experiment in which equal amounts of KCl and glucose were trapped in the liposome. After the addition of the polyene antibiotics we continuously monitored the release of K^+ and glucose. With filipin, no large differences in the efflux rate of K^+ and glucose are observed (Fig. 6). Amphotericin B and nystatin induce a fast K^+ leak and a slow (amphotericin B) or no (nystatin) efflux of glucose. In cholesterol free liposomes nystatin produces a K^+ and glucose leak which indicates that in the absence of cholesterol a non specific permeability change is observed. The effect of amphotericin B upon the turbidity of liposomes in which KCl, glucose or erythritol was trapped and which were brought in isotonic $MgCl_2$, KCl, glucose and erythritol is shown in Fig. 7. It is evident that K^+ and erythritol easily pass the membrane whereas the membrane is rather impermeable to Mg^{2+} and glucose. This conclusion holds for both the influx and efflux of these compounds.

Influence of sterol structure upon the filipin and amphotericin B induced permeability changes

Tables I and II show the filipin and amphotericin B induced K^+ leaks from cells grown in the presence of cholesterol, cholestanol, stigmasterol, stigmasterol, epicholesterol, epicholestanol and coprostanol [8]. Furthermore, the interactions between the sterols in the membrane and these antibiotics, as measured by difference spectroscopy [12, 13], changes in turbidity and glucose-6-phosphate dehydrogenase release are summarized. Incorporation of cholesterol, cholestanol or ergosterol in the membrane makes the cell sensitive towards the action of filipin and amphotericin B. Stigmasterol incorporated in the *A. laidlawii* cell membrane shows considerably less interaction with filipin and amphotericin than ergosterol. This would indicate that the sterol giving polyene sensitivity to yeast and fungi cells is mainly ergosterol. The

TABLE I

INTERACTION OF FILIPIN WITH *A. LAIDLAWII* CELLS GROWN ON DIFFERENT STEROLS

Test system as described in Fig. 1. Final filipin concentration 20 μg/ml. All data are means of experiments done on 3–5 different cultures.

Sterol present in the membrane	(3/1)* rel.	$\frac{dT}{dt}$ (% min)	% K^+ released after 10 min	% glucose-6-phosphate dehydrogenase released after 10 min
None	1.02	0.0	3	0
Cholesterol	2.18	2.1	40	32
Cholestanol	2.99	6.0	38	31
Ergosterol	2.01	1.5	18	18
Stigmasterol	1.56	0.0	5	2
Epicholesterol	1.47	0.7	10	1
Epicholestanol	1.16	0.0	3	0
Coprostanol	1.25	0.0	3	—

* (3/1) rel. is the ratio of the absorbances of Peaks 3 and 1 of filipin in the presence of the cells divided by the ratio of the absorbance of Peaks 3 and 1 of filipin in the buffer [12].

TABLE II

INTERACTION OF AMPHOTERICIN B WITH *A. LAIDLAWII* CELLS GROWN ON DIFFERENT STEROLS

For details see Table I.

Sterol present in the membrane	(3/1) rel.	$\frac{dT}{dt}$ (% min)	% K^+ released after 10 min	% glucose-6-phosphate dehydrogenase released after 10 min
None	1.06	0.0	5	0
Cholesterol	1.17	−2.7	100	0
Cholestanol	1.19	−1.6	60	0
Ergosterol	1.24	−23.0	100	0
Stigmasterol	1.06	−0.3	40	0
Epicholesterol	0.97	0.0	7	0
Epicholestanol	1.00	0.0	5	0
Coprostanol	1.11	0.0	5	0

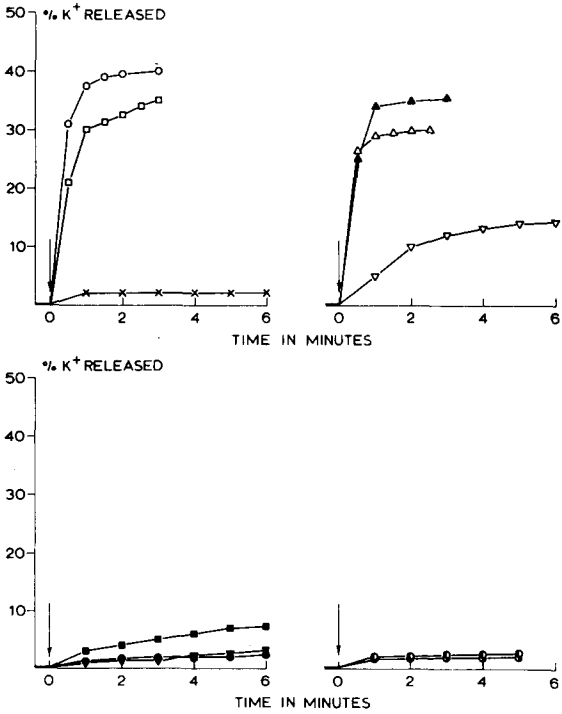

Fig. 8. Effect of amphotericin B upon the K^+ release at 25 °C from liposomes prepared from egg lecithin and 15.7 mole % of various sterols. Test system as described in Materials and Methods and the legend of Fig. 2. Amphotericin B was added at $t = 0$ such that the final concentration was 20 μg/ml. Sterols incorporated in the liposome were: ×, none; ○, cholesterol; ▽, 5α-androst-3β-ol; △, ergosterol; □, cholestanol; ▲, stigmasterol; ●, epicholesterol; ■, epicholestanol; ◧, coprostanol; ▼, 5α-androst-3α-ol; ◐, cholestane-4,6-diene-3-one; and ◑, cholesten-3-one.

3α-hydroxy isomers, epicholesterol and epicholestanol and the sterol with the bent-*cis* configuration A/B ring, coprostanol incorporated in the *A. laidlawii* cell membrane showed almost no interaction with filipin and amphotericin B.

In egg lecithin liposomes also we can incorporate the side chain less sterols 5α-androst-3α-ol and 5α-androst-3α-ol and the 3-ketosteroids cholestane-4,6-dien-3-one and cholesten-3-one [23]. The effect of incorporating 15.7 mole% of various sterols in the egg lecithin liposomes upon the amphotericin B induced K^+ leak is shown in Fig. 8. It is obvious that sterols having a 3β-OH group, a planar ring system and the hydrophobic side chain at C_{17}, which change the ultraviolet spectrum of filipin and amphotericin [12, 13], induce a strong K^+ release in the presence of amphotericin B. In the liposomal system but not in *A. laidlawii* cell membrane stigmasterol can interact with amphotericin and filipin (Fig. 8 and [12, 13]).

DISCUSSION

In this paper we compared the effects of filipin, amphotericin B, nystatin, etruscomycin and pimaricin upon the membrane permeability of *A. lailawii* cells and egg lecithin liposomes. In the absence of membranous cholesterol none of the polyene antibiotics had any significant effect upon the membrane permeability. In the presence of membranous cholesterol these antibiotics, with the exception of pimaricin, affected the membrane permeability.

Filipin, after the interaction with cholesterol, disrupts the membrane of *A. laidlawii* and a non-specific efflux of cytoplasmic components like glucose-6-phosphate dehydrogenase is observed (Figs 1–4, 6). Recently, it was demonstrated by Kataoka et al. [20] that filipin could also release trapped glucose-6-phosphate dehydrogenase from cholesterol containing lecithin liposomes. We propose that the permeability changes observed with filipin must be the result of a fragmentation of the membrane caused by the presence or formation of the 150–250 Å diameter filipin–cholesterol complexes which can be visualised with electronmicroscopy [5–7].

Amphotericin B, nystatin and etruscomycin alter the membrane permeability in a specific way. Large molecules like glucose-6-phosphate dehydrogenase, $NADP^+$, glucose 6-phosphate, Mg^{2+}, Ca^{2+} and sucrose do not permeate or permeate slowly (glucose) through the antibiotic modified membrane. Smaller molecules like K^+, Na^+, Rb^+, Li^+, ribose, xylose, erythritol and urea do permeate through the antibiotic modified membrane with a velocity which is dependent upon the size of the molecule (Figs 3, 5–7). The amphotericin B, nystatin and probably also the etruscomycin induced permeability changes observed in the membrane strongly suggest that these polyene antibiotics, after interacting with cholesterol, create aqueous pores in the membranes of *A. laidlawii* cells and egg lecithin liposomes. The amphotericin B produced pore must have a diameter similar to the hydrodynamic radius of glucose, a molecule which permeates slowly through the amphotericin B-modified membrane. Nystatin forms a pore with a somewhat smaller size because glucose does not pass this pore. The size of the etruscomycin formed pore probably has about the same dimensions as the amphotericin B and nystatin formed pore. These findings are in good agreement with the extensive studies on the amphotericin B and nystatin induced permeability changes in the black lipid model membrane from which it was also concluded that amphotericin B and nystatin form aqueous pores in cholesterol con-

taining membranes [3, 4, 22].

The stoichiometry of the polyene antibiotic–sterol complex formation producing the enhanced permeabilities of the *A. laidlawii* cell membrane can be estimated from the concentration of antibiotic producing the maximal K^+ efflux (filipin and amphotericin, 20 μg/ml; nystatin and etruscomycin, 50 μg/ml; Fig. 1) and the concentration of membranous cholesterol (4 μg/ml). The percentages of antibiotic binding to cholesterol in the *A. laidlawii* cell membrane are: filipin 48%, amphotericin B 15%, nystatin 12% and etruscomycin 44% [13]. If one assumes that all the cholesterol in the membrane interacts with the antibiotic then one can calculate that 0.7, 3.3, 1.6 and 0.3 molecules cholesterol bind per molecule of respectively, filipin, amphotericin B, nystatin and etruscomycin.

The amphotericin B and nystatin induced K^+ leak from cholesterol containing *A. laidlawii* cells was strongly dependent upon the antibiotic concentration (Fig. 1) as has also been noted for the haemolysis induced in erythrocytes [18] and the permeability changes induced in black lipid membranes [3, 4, 27]. This indicates that a number of molecules (6 estimated from Fig. 1, and 4.5–10 as measured for the black lipid membranes [3, 4, 27] are required for the formation of one pore.

The structure of the sterol molecule is an important parameter in the polyene antibiotic–sterol interaction. In ultraviolet spectroscopy [12, 13], fluorescence [25, 26] and monolayer studies [12, 13] it was demonstrated that for polyene antibiotic–sterol interaction in systems where only sterol was present, an intact steroid ring system was required. In mixed lipid–sterol liposomes and monolayers additional requirements for a 3β-OH group, a planar molecule and a hydrophobic side chain at C_{17} were observed [12, 13, 26]. In this study we demonstrated that the same structural requirements are found for the filipin and amphotericin B induced permeability changes in *A. laidlawii* cells and egg lecithin liposomes containing various sterols.

There is a striking correlation between the structural requirements of the polyene antibiotic–sterol interaction and the lipid-sterol interaction. For the lipid–sterol interaction measured in monolayers [11, 24], liposomes [23, 28] and *A. laidlawii* cells [8, 9] also a 3β-OH group, a planar molecule and a hydrophobic side chain were found to be prerequisites. The most obvious explanation for this finding is that the nature of the polyene antibiotic–sterol interaction is similar to the nature of the lipid–sterol interaction. That both interactions are primarily hydrophobic is well documented [11–13, 24–26, 28]. Furthermore the lipid–sterol interaction may be required to orient the sterol in such a way in the membrane that the polyene antibiotic can interact with it. In this way the structural requirement of the sterol molecules for its interaction with polyene antibiotics would be a reflection of the structural requirement of the lipid–sterol interaction.

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