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

II. TEMPERATURE DEPENDENCE OF THE POLYENE ANTIBIOTIC–STEROL COMPLEX FORMATION

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SUMMARY

1. The effect of filipin, amphotericin B, nystatin, etruscomycin and pimaricin upon the energy content of the gel → liquid-crystalline phase transition and upon the temperature dependence of the ATPase activity was studied in cholesterol-containing membranes of *Acholeplasma laidlawii* cells grown on elaidic acid. These experiments prove that the polyene antibiotics form complexes with cholesterol in the *A. laidlawii* cell membrane.

2. The effect of temperature and different fatty acid composition upon the polyene antibiotic–cholesterol interaction in *A. laidlawii* cells, lecithin liposomes and in an aqueous dispersion of microcrystalline dispersed cholesterol was investigated by (a) ultraviolet spectroscopy, (b) binding experiments, (c) freeze-etch electron microscopy, (d) K^+ permeability. From the results of these studies it is suggested that filipin interacts first with cholesterol throughout the membrane forming primary filipin–cholesterol complexes. These complexes subsequently rearrange in the membrane to 150–250 Å diameter aggregates which ultimately fragment the cell membrane. The results obtained for amphotericin B, nystatin and etruscomycin indicate that these antibiotics form much smaller complexes with cholesterol.

3. The primaricin–cholesterol complexes formed in the *A. laidlawii* and liposomal membranes do not affect membrane permeability. A possible explanation of the haemolytic, fungicidic and fungistatic properties of this polyene antibiotic is suggested.

INTRODUCTION

In the preceeding paper [1] we observed specific permeability changes induced by filipin, amphotericin B, nystatin, etruscomycin and pimaricin in cholesterol containing *Acholeplasma laidlawii* cells and egg lecithin liposomes. In order to correlate the observed permeability changes with the formation of polyene antibiotic–cholesterol complexes we investigated in this paper whether in the membrane of

A. laidlawii these antibiotics are capable of interacting with cholesterol in such a way that cholesterol is no longer available for the interaction with other lipids in the membrane. For this purpose we studied the effect of these antibiotics upon the phase transition in cholesterol containing *A. laidlawii* cell membranes. This was done directly with the differential scanning calorimeter and indirectly by studying the temperature dependence of the ATPase activity in the cell membrane. With the 'breaks' in the Arrhenius plots of this enzyme the cholesterol–lipid interaction can be demonstrated [2]. In order to obtain further insight into the role of the polyene antibiotic–sterol complex formation in the induced permeability changes and to explain the differences in mode of action of the various polyene antibiotics, we studied the influence of temperature, fatty acid composition and lipid phase transition upon the polyene antibiotic–cholesterol complex formation in the membranes of the *A. laidlawii* cells and liposomes of phospholipid and cholesterol.

EXPERIMENTAL

Materials and methods are described in the preceding publication [1]. 1,2-Dielaidoyl-*sn*-glycero-3-phosphorylcholine (18:1t/18:1t-phosphorylcholine) was synthesized as described before [3]. *A. laidlawii* cell membranes were isolated as described by Van Golde et al. [4]. Protein was determined according to Lowry et al. [5]. Fatty acid patterns and quantitation were determined by gas–liquid chromatography using 20:0 as a standard as described before [6]. The ATPase activity in the *A. laidlawii* cell membrane was measured as described before [2].

Phase transitions in *A. laidlawii* membranes were determined with a Perkin–Elmer DSC-2B calorimeter. Aliquots of membranes (1.5 mg protein) isolated from *A. laidlawii* grown on 0.06 mM elaidic acid (18:1t), 0.5 μCi [^{14}C]palmitic acid (spec. act. 55 Ci/mole) and 25 mg cholesterol per 1 of culture were suspended in 2.2 ml distilled water. 1 mg polyene antibiotic dissolved in 100 μl dimethyl formamide or dimethylsulfoxide was added, and the suspension was incubated at 37 °C for 10 min. Control membranes were incubated with 100 μl dimethylformamide. After the incubation the membranes were centrifuged at 37 000 $\times g$ for 1 h at 0 °C. 10 vol. % of glycol was added to the membrane pellet in order to reduce the freezing point of water. The pellet was carefully homogenized with a pasteur pipet at 0 °C. About 15 μl of this cell suspension was sealed in the sample pan. The sample was scanned at range 0.5 at a rate of 5 or 10 °C/min at least 4 times up to 40 °C (below the temperature of the protein denaturation) and at least 3 times up to 70 °C at which temperature protein denaturation occurs [7, 8]. Protein denaturation did not influence the heat content of the phase transition of the membrane lipids. After the scans were made the sample pans were opened and the amount of ^{14}C radioactivity in the pan was determined. On an aliquot of the membrane suspension the amount of total cholesterol-free lipid and ^{14}C radioactivity was determined. With these data the amount of total lipid (without cholesterol) present in the sample pan could be calculated. The calorimeter was calibrated with water, cyclohexane and naphthalene as standards.

RESULTS

Polyene antibiotic–cholesterol complex formation in the A. laidlawii cell membrane

Cholesterol incorporation in the *A. laidlawii* cell membrane decreases the

TABLE I

EFFECT OF THE POLYENE ANTIBIOTICS UPON THE ENERGY CONTENT OF THE PHASE TRANSITION OF THE LIPIDS IN THE MEMBRANE OF 18 : 1t GROWN *A. LAIDLAWII* CELLS

Polyene antibiotic	Energy content of the phase transition in cal per g of total cholesterol-free lipid ±S.D.	Number of determinations
None	8.2±0.4	9
Filipin	10.6±0.4	4
Amphotericin B	9.6±0.4	8
Nystatin	11.4±0.5	5
Etruscomycin	13.5±1.0	5
Pimaricin	12.8±0.6	5

energy content of the phase transition of the lipids in membranes of elaidic acid (18:1t) grown cells by about 32%, indicating that cholesterol interacts with the lipids in the membrane [6]. Addition of the polyene antibiotics caused an increase in the energy content of the phase transition (Table I). These results indicate that all the polyene antibiotics tested can complex the cholesterol in the *A. laidlawii* cell membrane in such a way that it is no longer available for the interaction with other lipids. The differences in the increase of the energy content of the phase transition for the various polyene antibiotics (Table I) might be caused by differences in binding of the various polyene antibiotics to the membrane [9] or by differences in stoichiometry of the polyene antibiotic–cholesterol complexes [1, 9, 10]. The maximum increase in energy

TABLE II

EFFECT OF THE POLYENE ANTIBIOTICS UPON THE BREAK IN THE ARRHENIUS PLOT OF THE ATPase ACTIVITY IN MEMBRANES ISOLATED FROM *A. LAIDLAWII* CELLS GROWN ON 18 : 1t IN THE PRESENCE OR ABSENCE OF CHOLESTEROL

Membranes of cells grown on 0.06 mM 18 : 1t with or without 25 mg cholesterol per l of culture were isolated and suspended in distilled water. 10 ml of this membrane suspension (1 mg membrane protein) was incubated at 25 °C for 30 min with 1 mg filipin (10 mg/ml dimethylformamide). Control membranes were incubated with 100 µl dimethylformamide. Immediately after the incubation 0.65-ml aliquots were taken for the determination of the ATPase activity at various temperatures. The variation in duplicate experiments for the determination of the breaks in the Arrhenius plots was less than 0.3 °C.

Membranes	Added polyene antibiotic	Temperature of the break in the Arrhenius plot of the ATPase activity (°C)
−cholesterol	−	15.5
+cholesterol	−	≦ 10.0
+cholesterol	Filipin	15.8
+cholesterol	Amphotericin B	15.4
+cholesterol	Nystatin	13.4
+cholesterol	Etruscomycin	13.4
+cholesterol	Pimaricin	13.4

content which can be obtained if all the cholesterol is complexed in the *A. laidlawii* cell membrane can be calculated using the data from [6] to be 12.1 cal/g. Etruscomycin and pimaricin increase the energy content up to this value (Table I) which indicates that under the conditions of this experiment all the cholesterol present in the membrane is complexed by these polyene antibiotics.

The interaction between cholesterol and the membrane lipids of *A. laidlawii* can also be demonstrated by measuring the temperature dependence of the ATPase activity in the cell membrane [2]. In the absence of cholesterol the Arrhenius plot of the ATPase activity shows a discontinuity at 15.5 °C in good agreement with the onset of the calorimetrically determined phase transition [6]. Cholesterol shifts the break towards below 10 °C. All the polyenes shift the discontinuity in the Arrhenius plot toward the temperature observed in the absence of cholesterol; in the case of filipin and amphotericin B, we observed that the temperature of the breaks coincided with the temperature of the break in the membranes of cholesterol-free cells

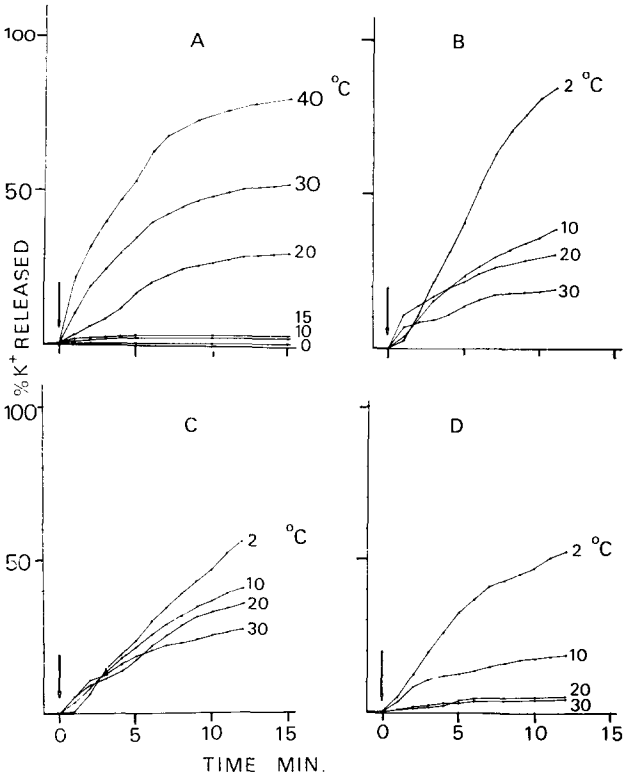
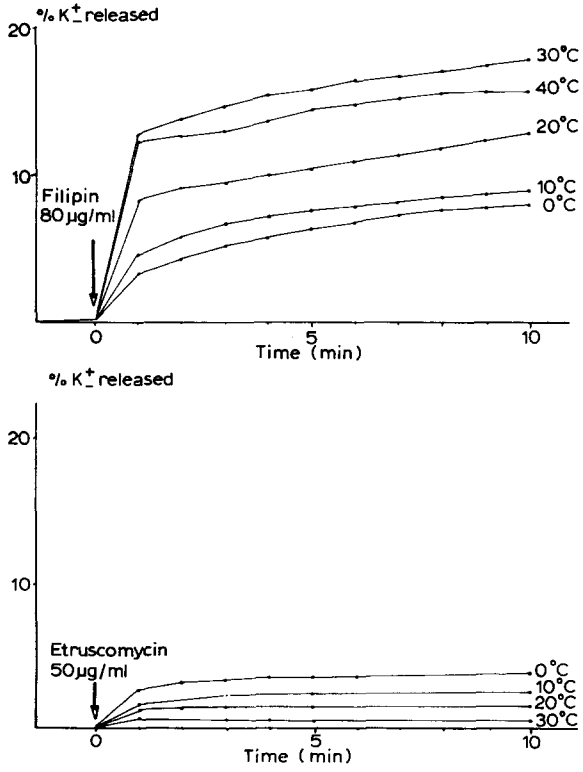


Fig. 1. Temperature dependence of the polyene antibiotic induced K^+ leak from cholesterol and oleic acid grown *A. laidlawii* cells. Cells were grown on 0.06 mM 18 : 1c and 25 mg cholesterol per l of culture. 100 μl washed cells (300 μg cell protein) were brought in 5 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) to the desired temperature. After the determination of the passive K^+ leak the antibiotics were added such that the final concentrations were 20 μg/ml for filipin (A), nystatin (C) and etruscomycin (D) and 0.8 μg/ml for amphotericin B (B). All leaks are corrected for the passive efflux of K^+ from the cells. Under these conditions the polyene antibiotics did not produce any measurable efflux of K^+ from the cholesterol free *A. laidlawii* cells.

(Table II). This experiment confirms that the polyene antibiotics complex with cholesterol in the *A. laidlawii* membrane and restrict its interaction with other lipids.

Temperature dependence of the polyene antibiotic induced K^+ leak

The phase transition in the membranes of *A. laidlawii* cells grown on cholesterol and oleic acid occurs below 0 °C [11]. Above this temperature the fatty acid chains are in the liquid–crystalline state. Fig. 1 demonstrates the filipin, amphotericin B, nystatin and etruscomycin induced K^+ leak from these cells in the temperature range 0–40 °C. The rate and extent of the filipin induced K^+ leak decreases at lower temperatures, and below 15 °C no effect of filipin upon the membrane permeability could be detected. The other polyene antibiotics show a reversed temperature dependence of the induced K^+ leak (Fig. 1). That is, at high temperature little (amphotericin B and nystatin) or no (etruscomycin) K^+ efflux was observed. Decreasing the temperature markedly stimulates the induced K^+ leak, such that the maximal effect is observed at 0 °C. Pimaricin had no effect upon the K^+ permeability also at 0 °C. In the liposomal system the filipin-induced K^+ leak also decreases with decreasing temperature, although temperature dependence is much less pronounced than in *A. laidlawii* cells (Fig. 2a). Filipin at 0 °C still induces a significant K^+ leak from the liposomes. The amphotericin B, nystatin and etruscomycin induced leaks from the liposomes all increase with decreasing temperatures (Figs 2 a–c).



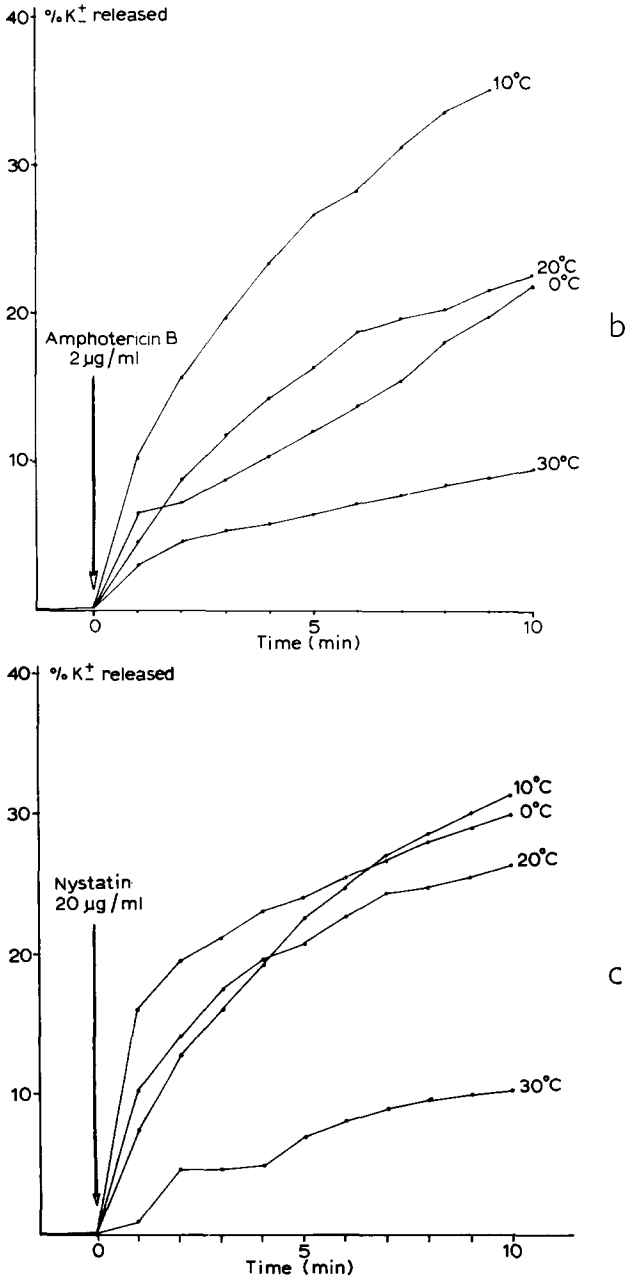


Fig. 2. Temperature dependence of the filipin (A) and etruscomycin (A), amphotericin B (B) and nystatin (C) induced K^+ leak from cholesterol containing egg lecithin liposomes. 50 μg egg lecithin liposomes containing no or 15.7 mole % cholesterol were brought in 5 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) at the desired temperature. After the determination of the passive K^+ leak the antibiotics were added to the concentrations indicated in the figure. The % K^+ released plotted in the figure is corrected for the passive K^+ leak from the liposomes and the antibiotic induced K^+ leak from cholesterol free liposomes [1].

Temperature dependence of the polyene antibiotic–cholesterol complex formation

Table III demonstrates that the interaction of both filipin and amphotericin B with cholesterol dispersed in buffer, as measured by the increase of the absorbance ratio of Peaks 1 and 3 [10], is maximal at 0 °C and decreases with increasing temperature, indicating that at higher temperatures the antibiotic–cholesterol complex dissociates. The interaction of filipin with cholesterol present in egg lecithin liposomes was virtually independent of the temperature. The change in the ultraviolet spectrum of amphotericin B in the presence of cholesterol-containing liposomes was too small to draw a conclusion.

TABLE III

TEMPERATURE DEPENDENCE OF THE FILIPIN–CHOLESTEROL AND AMPHOTERICIN B–CHOLESTEROL INTERACTION IN CHOLESTEROL CONTAINING EGG LECITHIN LIPOSOMES AND IN AN AQUEOUS DISPERSION OF CHOLESTEROL

Test system: In a tube to 1.0 ml 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5), 5 μl 15.7 mole % cholesterol containing egg lecithin liposomes (15 μg cholesterol) or 15 μl ethanolic solution of cholesterol (1 mg/ml) was added. The mixture was vortexed and brought on the desired temperature. 20 μg filipin or 2 μg amphotericin B was added and after 5 min the ultraviolet spectrum was recorded.

	(3/1) rel*		
	0 °C	20 °C	40 °C
Filipin			
in buffer	1.00	1.00	1.00
cholesterol dispersed in buffer	2.90	2.70	2.44
cholesterol containing liposomes in buffer	2.06	2.09	2.09
Amphotericin B			
in buffer	1.00	1.00	1.00
cholesterol dispersed in buffer	1.84	1.45	1.45

* (3/1) relative is the ratio of the absorbances of Peaks 3 and 1 of filipin or amphotericin B in the presence of liposomes or cholesterol dispersion divided by the ratio of the absorbances of Peaks 3 and 1 of filipin and amphotericin B in buffer.

The filipin –cholesterol complex formation in the membranes of intact *A. laidlawii* cells was strongly temperature dependent (Fig. 3A). In marked contrast to the filipin–cholesterol complex formation in an aqueous dispersion of cholesterol we observed a decreased interaction at lower temperatures. At 0 °C almost no interaction could be detected. When cells after interaction with filipin at 40 °C, peak ratio (3/1) rel 1.5 were cooled down to 0 °C the peak ratio (3/1) rel increased to 1.7. This strongly indicates that although the filipin–cholesterol complex is very stable at 0 °C this complex cannot be formed at this temperature in the membrane of the intact *A. laidlawii* cell. This conclusion is also supported by the finding that the filipin–cholesterol complexes which can be visualized by freeze-etch electronmicroscopy [12] are not found in cholesterol containing *A. laidlawii* cells at 0 °C. When cells which had been incubated with filipin at 0 °C were heated to 37 °C for 5 min filipin–cholesterol complexes were observed in the membrane (electron micrographs not shown, results were identical as described in ref. 12, see also the succeeding paper [13]).

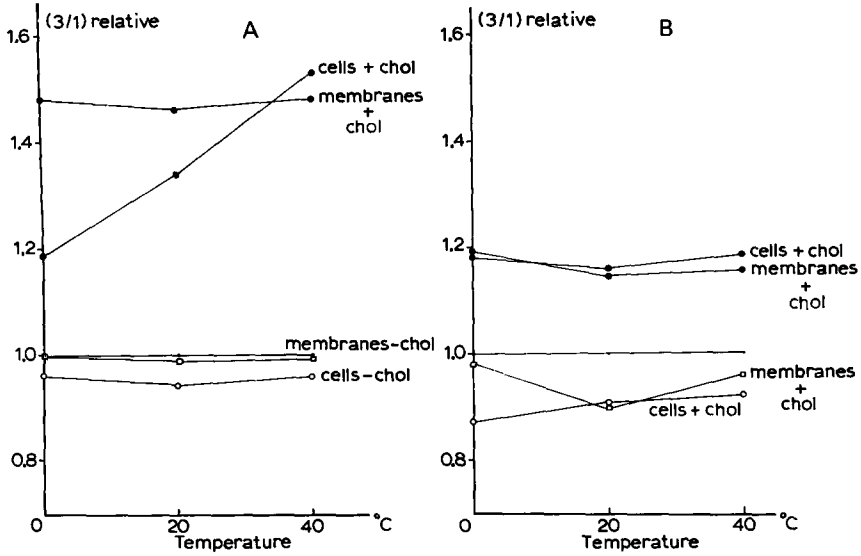


Fig. 3. Temperature dependence of the interaction of filipin, and amphotericin B with cholesterol in the *A. laidlawii* cell membrane. Cells were grown on 0.06 mM 18 : 1c and 25 mg cholesterol (containing 4 μCi [1-^{14}C]cholesterol) per l of culture. From part of the cells membranes were isolated. Washed cells were suspended in 100 mM $CaCl_2$–10 mM Tris–HCl buffer (pH 7.5) to a concentration of 3 mg cell protein per ml. Membranes were suspended in water such that the concentration of [^{14}C]cholesterol was identical for the cells and the membranes. 100 μl cell or membrane suspension was added to 1 ml $CaCl_2$ buffer. The solution was brought to the desired temperature and 20 μg filipin (A) or 2 μg amphotericin B (B) was added.

The interaction between filipin and cholesterol in isolated *A. laidlawii* membranes was not very much temperature dependent in contrast to the interaction with cholesterol present in the membrane of intact cells. Appararently, in isolated membranes filipin–cholesterol complexes can be formed at low temperatures.

The amphotericin B–cholesterol complex formation was identical in both the isolated membranes and the intact cells and exhibited no significant temperature dependence (Fig. 3B). Thus at all temperatures most, if not all the cholesterol is available for complex formation with amphotericin B.

Temperature dependence of the binding of filipin to the A. laidlawii cell membrane

In order to determine whether the cholesterol in the intact *A. laidlawii* cell membrane is available for interaction with filipin at lower temperatures, we studied the temperature dependence of the binding of this antibiotic. Table IV demonstrates that filipin binds to cholesterol-containing *A. laidlawii* cells as strongly at 0 °C as at 20 and 40 °C. This indicates that filipin can interact with cholesterol in the membrane at 0 °C but that this interaction leads neither to the formation of spectroscopically detectable complexes nor to K^+ release. About twice as much filipin was bound to the isolated membranes than to the intact cells. In the intact cell membrane 1 molecule filipin was present per 3 molecules of cholesterol whereas in the isolated membrane 1 filipin molecule was present per 1–2 molecules of cholesterol. From the results of a similar experiment performed with the other polyene antibiotic at 20 °C we calculated

TABLE IV

TEMPERATURE DEPENDENCE OF THE BINDING OF FILIPIN TO CHOLESTEROL CONTAINING *A. LAIDLAWII* CELLS AND MEMBRANES

The experimental conditions of this experiment were identical as described in the legend of Fig. 3. 1.0 ml buffer contained 20 μg filipin and 9.9 μg membranous cholesterol. After the 5 min incubation of filipin with the cells or membranes, the reaction mixture was centrifuged for 30 min at 37 500 $\times g$ at the same temperature at which the incubation had been carried out. To 0.25 ml supernatant 0.25 ml water and 0.5 ml methanol was added and the amount of filipin was determined by measuring $A_{337\ nm}$. The absorbance of filipin in water–methanol (1 : 1, v/v) at this wavelength is linear with the filipin concentration [10].

	μg filipin bound			mole cholesterol / mole filipin bound		
	0 °C	20 °C	40 °C	0 °C	20 °C	40 °C
Cells +cholesterol	6.3	7.5	6.3	2.9	3.0	3.2
Cells −cholesterol	0.4	1.3	1.2	—	—	—
Membranes +cholesterol	8.3	15.2	14.6	2.2	1.2	1.2
Membranes −cholesterol	0.6	1.0	0.2	—	—	—

that the number of cholesterol molecules bound per mole of antibiotics in the isolated *A. laidlawii* cell membranes was 0.7, 1.6 and 1.5 for, respectively, amphotericin B, etruscomycin and pimaricin.

Effects of the phase transition of the membrane lipids upon the polyene antibiotic–cholesterol interaction

A. laidlawii cells grown on elaidic acid and cholesterol show a transition in the membrane lipids over the range 10-37 °C [6]. In Fig. 4 the polyene antibiotic

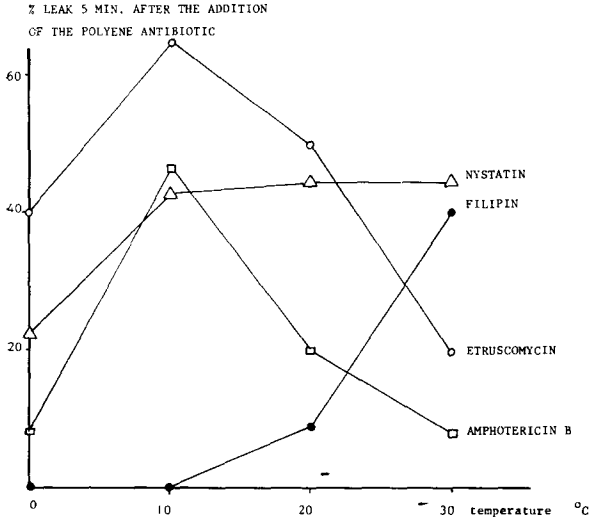


Fig. 4. Temperature dependence of the polyene antibiotic induced K^+-leak from cholesterol and elaidic acid grown *A. laidlawii* cells. Cells were grown on 0.06 mM 18 : 1t and 25 mg cholesterol per l of culture. Experimental procedure as described in the legend of Fig. 3.

induced K^+ leak from these cells is presented. From 10 to 30 °C we observed temperature dependence similar to that for oleic acid grown cells (Fig. 1). With decreasing temperatures the K^+ leak was reduced in the presence of filipin. The amphotericin B and etruscomycin induced K^+ leaks increased whereas the nystatin induced K^+ leak remained nearly constant with decreasing temperature. Below the starting temperatures of the phase transition (10 °C) the amphotericin B, nystatin and etruscomycin induced K^+ leak decreased (Fig. 4); nevertheless, significant transport occurs below the transition temperature. We must emphasize that these measurements can be performed with *A. laidlawii* cells because they are stable at temperatures below the transition and no spontaneous loss of K^+ is observed [11]. At none of the temperatures tested (also below the transition temperature of the membrane lipids) the polyene antibiotics did induce a measurable K^+ leak from *A. laidlawii* cells grown on elaidic acid without cholesterol. At a higher amphotericin B concentration (20 μg/ml) the K^+ leak at 0 °C from the 18:1t/cholesterol grown cells was identical to the leaks at 10 and 20 °C.

The influence of the phase transition of the membrane lipids upon the polyene antibiotic–cholesterol complex formation can also be studied spectroscopically. Complex formation can be followed not only by an increase in the absorbance ratio (3/1) but also as a decrease in the absorbance of Peak 1. The halftime of the decrease in the absorbance at 347.5 nm (absorbance Peak 1 of filipin [10]) after the addition of

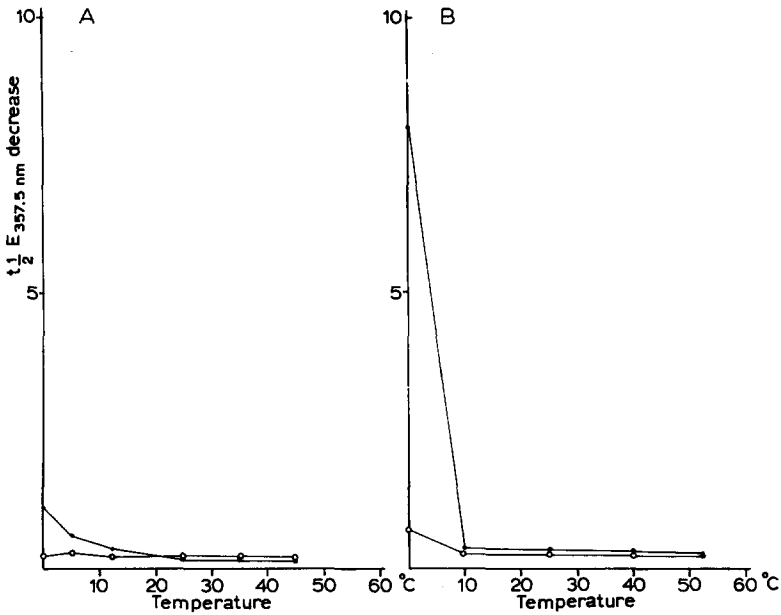


Fig. 5. Temperature dependence of the velocity of the filipin–cholesterol interaction in the membranes of *A. laidlawii* cells and liposomes. Cells were grown on 0.06 mM 18 : 1c and 0.06 mM 18 : 1t with 25 mg cholesterol per l of culture (A). Liposomes were prepared from egg lecithin and 18 : 1t/18 : 1t phosphatidylcholine in the presence of 15.7 mole% cholesterol (B). Experimental conditions as described in the legends of Table III and Fig. 2. 15 s after the addition of the antibiotic the extinction at 357.5 nm was recorded as a function of the time until the absorbance remained constant (about 10 min). ○–○, membranes without cholesterol; ●–●, membranes with cholesterol.

cholesterol-containing cells or liposomes to a filipin solution is a measure of the rate of the filipin–cholesterol complex formation. In Fig. 5 such rates of the filipin–cholesterol complex formation in 18:1c/cholesterol containing liposomes prepared from egg phosphatidylcholine and 18:1t/18:1t–phosphatidylcholine are compared. The transition temperature of 18:1t/18:1t–phosphatidylcholine is about 10 °C [9]. It is obvious that the halftimes of the spectroscopically measured changes with cells and liposomes are small at all temperatures when the membrane lipids are in the liquid–crystalline state. As soon as the membrane lipids which are not interacting with cholesterol become in the gel state the velocity of the complex formation decreases. This effect is most clear in case of the liposomes. With cells, this effect is less pronounced as a result of the low quantity of filipin–cholesterol complex at lower temperatures (see Fig. 3). From Fig. 5 it is evident that the halftime of the filipin–cholesterol complex formation in a membrane with the fatty acid chains of the membrane lipids in the liquid–crystalline state is in the order of 15 s or less. In the K^+ leak experiments performed on *A. laidlawii* cells with membrane lipids in the liquid–crystalline state ([1], cf. also Fig. 1) we observed that the halftime of the induced K^+ leak was in the order of 60 s or larger, suggesting that complex formation precedes membrane fragmentation.

DISCUSSION

In this paper we have demonstrated that filipin, amphotericin B, nystatin, etruscomycin and pimaricin shield cholesterol from its interaction with other lipids in the *A. laidlawii* cell membrane. Apparently then, the polyene antibiotic–cholesterol interaction must be stronger than the lipid–cholesterol interaction. This conclusion is also supported by our previous studies on polyene antibiotic complex formation in model membranes of lecithin liposomes [9, 10].

The polyene antibiotic–cholesterol complex formation exhibited a pronounced temperature dependence. The filipin–cholesterol complex formed in aqueous solutions of microcrystalline dispersed cholesterol showed the strongest interaction as detected spectroscopically at 0 °C. The complex dissociates with increasing temperatures suggesting that the interaction forces in the complex are mainly hydrophobic (cf. [10] and Table III). In the membranes of *A. laidlawii* cells, however, no filipin–cholesterol complex formation was observed at low temperatures (0–15 °C). Under these conditions, and in contrast to temperatures greater than 15 °C, there were no changes in the ultraviolet absorption spectrum, no aggregate formation detectable by freeze-etch electron microscopy, and no release of K^+ following the addition of filipin to the cholesterol-containing cells. However, at these same temperatures we did observe a binding of filipin to intact cholesterol-containing *A. laidlawii* cell membranes which was comparable to the binding at higher temperatures. These experiments suggest first that the filipin–cholesterol complex detected by spectroscopic methods is identical to the filipin–cholesterol complex observed by electron microscopic methods. Furthermore the mechanism of the complex formation which emerges from these findings is that filipin interacts first with cholesterol present all over the membrane. These primarily formed filipin–cholesterol complexes (detected in the binding experiments) tend to aggregate. The presence of the large filipin–cholesterol aggregates finally leads to the disruption of the cell membrane. The aggregation proceeds via lateral

diffusion of these primary complexes in the membrane. This diffusion step is dependent upon the mobility of the fatty acid chains. Thus, the formation of filipin–cholesterol complexes at lower temperatures must be diminished due to low thermal motion. In this regard we found a markedly decreased velocity of filipin–cholesterol complex formation below the transition temperature of the membrane lipids (Fig. 5). The filipin–cholesterol complexes are more easily formed in the isolated cholesterol containing *A. laidlawii* cell membrane at lower temperatures than in the membrane of the intact cell (Fig. 3). This suggests that for the formation of the complexes cholesterol is needed at both sides of the membrane. In the liposomal system the formation rate of the spectroscopically detected filipin–cholesterol complexes was not very much decreased at low temperatures; moreover, considerable amounts of aggregates could be detected with freeze-etch electron microscopy in the membranes at these temperatures (unpublished observations). At 0 °C a significant complex formation (Table III) and induced K^+ leak (Fig. 2a) was observed .The lateral diffusion of the primary complexes at 0 °C is apparently higher in the liposomal membrane than in the biological membrane, although the fatty acid composition is comparable. One explanation of this difference may be that membrane proteins in the *A. laidlawii* cell membrane are responsible for a decreased lateral diffusion of the primary filipin–cholesterol complexes. An analogous situation described by Dupont et al. [13] is the greater hysterisis of the gel→liquid–crystalline phase transition of lipids in biological membranes as compared to liposomal membranes. These authors suggested that the membrane proteins were interfering with the segregation of the gel phase.

The amphotericin B–cholesterol complex formation in an aqueous dispersion of crystalline cholesterol is found to be maximal at 0 °C (Table III). Similar results were noted in interactions between amphotericin B, nystatin, etruscomycin and cholesterol in liposomes and *A. laidlawii* cells. For the formation of the large filipin–cholesterol complex in the membrane the primary filipin–cholesterol complexes have to diffuse over a longer distance than in the case of the other polyenes where the small pores [1, 14, 15] are composed of a relative small number of molecules. This explains why the membrane of the *A. laidlawii* cell showed no decreased permeability in the presence of amphotericin B, nystatin and etruscomycin at lower temperatures in contrast to filipin. However, below the transition temperature of the lipids in the *A. laidlawii* cell we observed that the amphotericin B, nystatin and etruscomycin induced K^+ leak decreased. Apparently, the penetration of amphotericin B in the membrane becomes more difficult or the formation rate of the pore is decreased because of the gel state of the fatty acids. Hsuchen and Feingold [16] recently observed in contrast to our experiments performed on the *A. laidlawii* cells that below the transition temperature of the lipids these antibiotics induced a strong glucose leak from cholesterol-free liposomes derived from dipalmitoyl and distearoyl lecithin. Cholesterol incorporation into these liposomes decreased the polyene antibiotic induced glucose release. They suggested that an ordered state of the membrane is required for a disruptive interaction between polyene antibiotics and membranes. Their results, however, can also be explained in a different way. In their assay the glucose leak from liposomes loaded with glucose and suspended in isotonic NaCl was measured after the addition of amphotericin B and nystatin. Since these antibiotics create aqueous pores in the membrane which will permit only a small glucose efflux and a much faster Na^+ influx a swelling of the liposomes will occur as described in the preceeding paper [1].

Since the membranes are in the gel state swelling might cause a breaking of the liposomes [6, 11] producing a strong glucose leak. Cholesterol will prevent the crystallization of the fatty acid chains and might so protect the membrane against this swelling induced glucose leak [6, 11].

Pimaricin does not affect the membrane permeability of cholesterol containing *A. laidlawii* cells and egg lecithin liposomes [1]. Nevertheless, this polyene antibiotic binds to cholesterol in these membranes (Tables I and II and [9]). But the pimaricin–cholesterol complex cannot form large aggregates like filipin nor pores like amphotericin B, nystatin and etruscomycin. Pimaricin, however, does induce haemolysis in erythrocytes [17] and has fungistatic and fungicidic properties [18]. Since cholesterol is an essential structural component of the erythrocyte and fungal cell membrane but not of the *A. laidlawii* cell membrane, we suggest that primary action of pimaricin is the complexation of cholesterol. It is known that decreasing the cholesterol content of the erythrocyte membrane renders it more fragile [19].

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