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THE ACTION OF POLYENE ANTIBIOTICS ON LIPID BILAYER MEMBRANES IN THE PRESENCE OF SEVERAL CATIONS AND ANIONS

H. VAN ZUTPHEN, R. A. DEMEL, A. W. NORMAN* AND L. L. M. VAN DEENEN

*Laboratory of Biochemistry, State University of Utrecht, Vondellaan 26, Utrecht (The Netherlands) and *Department of Biochemistry, University of California, Riverside, Calif. 92502 (U.S.A.)*

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SUMMARY

1. Filipin complex, filipin II, filipin III, nystatin, etruscomycin, and pimaricin at concentrations of 10^{-5} M were able to disrupt bimolecular lipid films containing lecithin and cholesterol in a 1:1 molar ratio.

2. The above antibiotics were not able to disrupt lecithin bilayer membranes at concentrations of $4.0 \cdot 10^{-5}$–$6.25 \cdot 10^{-5}$ M. However, filipin complex and nystatin at higher concentrations (10^{-4}–10^{-2} M) affected lecithin bilayers in the absence of cholesterol.

3. Derivatives of filipin complex (perhydrofilipin and irradiated filipin), which have little or no biological activity, affected neither lecithin–cholesterol, nor lecithin bilayer membranes at concentrations of $4.0 \cdot 10^{-5}$ M, but disrupted both types of bilayer films at concentrations of 10^{-3} M.

4. Filipin II, filipin III, filipin complex, and amphotericin B could reduce d.c. resistances in decreasing order when added to one side of lecithin–cholesterol bilayer membranes generated in 0.1 M NaCl solutions. Filipin I, filipin IV, nystatin, pimaricin, and etruscomycin did not show this property. The antibiotics did not affect the d.c. resistances of lecithin bilayers.

5. Addition of nystatin to one side of lecithin–cholesterol bilayer membranes generated in 0.1 M NaCl solutions, did not affect the electrical resistances. When added to both sides of these bilayers, however, nystatin reduced the d.c. resistance appreciably.

6. When added to the film-forming solution, filipin III, filipin II, filipin complex, and nystatin were able to reduce the d.c. resistance of lecithin–cholesterol bilayer membranes in the order listed.

7. The d.c. resistance of lecithin–cholesterol bilayers generated in 0.1 M NaCl and treated with either filipin complex, filipin II, filipin III, nystatin, or pimaricin was much lower (10^4–10^5 $\Omega \cdot cm^2$) in the presence of Ca^{2+}, but was not affected in the presence of a variety of other cations and anions. Transference number measurements showed that lecithin–cholesterol bilayers, when treated with the polyenes mentioned, become cation-selective, particularly affecting the transference numbers of Ca^{2+}.

8. The d.c. resistance of lecithin–cholesterol bilayers generated in 0.1 M NaCl and treated with amphotericin B, was extremely low in the presence of NO_3^-, SO_4^{2-}, or HPO_4^{2-}, but not in the presence of Ca^{2+} or other cations. Transference number

measurements demonstrated that amphotericin B renders lecithin–cholesterol bilayers anion-selective.

INTRODUCTION

The results of several research groups[1–3] have indicated that polyene antibiotics, such as filipin, nystatin, amphotericin B and etruscomycin can affect the membrane permeability and finally cause membrane damage of sensitive organisms. Experiments on biological systems suggest that sterols are the unique complexing components of polyene-sensitive cells. Direct evidence that the presence of sterols in the cell membrane is a necessary prerequisite for the polyene sensitivity is provided by experiments with the pleuropneumonia-like organism, *Mycoplasma laidlawii*[4,5].

Filipin and amphotericin B were found to inhibit growth and to cause lysis of these organisms only when they were cultured in the presence of cholesterol. Also experiments with monomolecular lipid layers[6] demonstrated that polyene antibiotics preferentially interacted with cholesterol, at least when low molar ratios of antibiotic/lipid were used. However, at high molar ratios antibiotic/lipid, filipin, nystatin and pimaricin were found to increase the surface pressure of lecithin monolayers, in the absence of cholesterol[7].

WEISSMANN AND SESSA[8], using liposomes as model membranes, found that filipin and etruscomycin are able to cause leakage of various markers from liposomes prepared with lecithin alone, whereas nystatin and amphotericin B preferentially disrupt those which contain cholesterol. These authors added the antibiotics to the liposomes at concentrations between 10^{-4} and 10^{-3} M.

Recently KINSKY *et al.*[10] using a sensitive spectrophotometric assay to study the release of trapped glucose marker demonstrated that the presence of sterol increased the permeability of liposomes at much lower concentrations of filipin (10^{-6}–10^{-5} M).

According to observations of BERGY AND EBLE[11], filipin can be resolved into at least 4 components. The original filipin complex appeared to be composed of a component "filipin I", itself a mixture of polyenes, and the filipins II, III and IV, which were three pure pentaenes. The last mentioned, account for 96 % of the crystalline starting material.

SESSA AND WEISSMANN[12] showed that filipin III, which is the major (53 %) component of the complex, has a strong affinity for liposomes prepared with cholesterol, while filipin II (25 % of the complex) disrupts liposomes whether or not cholesterol is present. The filipins I and IV had no effect on these artificial membranes. The authors[12,13] concluded that the effects of filipin III predominate at low concentrations (10^{-9}–10^{-5} M, such as those employed in studies on biological membranes and monolayers) and the effects of filipin II at higher concentrations (10^{-4}–10^{-3} M). However, the monolayer experiments of DEMEL[14] indicated that filipin II also interacts preferentially with cholesterol at low antibiotic/lipid ratios. This filipin component appeared to be somewhat less active than filipin III in increasing the surface pressure of lecithin–cholesterol monolayers.

In earlier reports[15,16] we have shown that neither filipin complex, nor nystatin, at a concentration of approx. $4 \cdot 10^{-5}$ M, have any effect on the stability of lecithin

bilayers. However, membranes formed from equimolar solutions of lecithin and cholesterol were disrupted after addition of the antibiotics. The lecithin–cholesterol bilayers seemed to be less stable (*i.e.* had shorter survival times) in the presence of filipin than in the presence of nystatin. In these experiments we have adopted a film duration of 1 h or more as a criterion for membrane stability.

Considering the experiments with biological and model membranes discussed thus far, the conclusion can be drawn that the effects caused by low concentrations of polyenes (*i.e.* lysis of natural membranes, increase in the surface pressure of lipid monolayers and disruption of liposomes and lipid bilayers) are determined by the affinity of these compounds for sterol.

In contrast with our results[15,16], ANDREOLI AND MONAHAN[17], using bilayer membranes formed from lipids extracted from sheep erythrocytes and cholesterol, found that nystatin (as well as amphotericin B) had no detectable effect in the concentration range 10^{-8}–$2 \cdot 10^{-5}$ M, on the stability of these bilayers, but considerably reduced their d.c. resistances. The extent of this reduction appeared to depend on the antibiotic concentration and on the presence of sterol. The antibiotics had no effect on the d.c. resistance of films that did not contain cholesterol above a critical threshold value. Filipin complex disrupted the sheep erythrocyte lipid–cholesterol bilayers, in agreement with our findings[15,16]. FINKELSTEIN AND CASS[18], using bilayers prepared from ox brain lipids, confirmed the observations of ANDREOLI AND MONAHAM[17].

It has been known for several years that sublytic concentrations of polyene antibiotic can increase cation permeability in mammalian erythrocytes[19]. Recently, NORMAN and co-workers[20,41] observed that filipin is capable of increasing the *in vitro* permeability to Ca^{2+} of intestinal mucosal cells obtained from rachitic chicks, but not of mucosal cells of normal chicks. The effect appeared to be remarkably specific for Ca^{2+}. These authors also found that nystatin and amphotericin B were far less effective in specifically increasing the permeability of ileal segments from vitamin D-deficient chicks to Ca^{2+} transport.

Various studies have provided evidence that polyene antibiotics also influence the permeability of lipid bilayers. LIPPE[21] has demonstrated that amphotericin B increases thiourea permeability across cholesterol–dodecane bilayers, whereas it does not affect thiourea permeability across dioleoyl lecithin–decane films. FINKELSTEIN AND CASS[18,22] have reported that nystatin increases the permeability of ox brain lipid bilayers to water and certain small organic molecules (*e.g.* urea, glycerol, ethylene glycol, propionamide, erythritol) but not to sucrose or glucose. ANDREOLI *et al.*[23] have published data showing that amphotericin B has similar effects on sheep erythrocyte lipid films. Both laboratories[18,22,23] observed that bilayer permeability to water and non-electrolytes was not affected by the antibiotics when cholesterol was absent from the membranes. Nystatin and amphotericin B were also shown to alter cholesterol-containing bilayers from cation-selective to anion-selective structures[17,18].

In contrast to the effects of these antibiotics, which contain polar substituents, the neutral polyene filipin disrupted the architecture of the cholesterol-containing bilayers so that no effect on their permeability properties could be determined.

The above findings stimulated us to examine the action of polyene antibiotics on the stability and the electrical resistance of lipid bilayer membranes and to investigate whether the effects measured would depend on the concentration of chol-

esterol in the film-forming solution, the concentration of antibiotic added, and the method of addition of the polyenes. Considering the action of filipin on intestinal Ca^{2+} absorption, it seemed of interest to study the effects of various cations and anions on the d.c. resistance of antibiotic-treated bilayer membranes. Additional experiments were done to find out whether the transference numbers of these ions in antibiotic-treated bilayers would differ from those in untreated membranes.

MATERIALS AND METHODS

Egg lecithin was isolated according to established procedures[9]. The purity was routinely examined by chromatography on silica gel plates with chloroform–methanol–water (65:35:4, by vol.). (1,2-Dioleoyl)-3-lecithin was synthesized and purified according to methods described previously[24]. Cholesterol was purchased from Fluka AG, Switzerland, *n*-decane from Dr. Theodor Schuchardt, G.M.B.H. and Co., Germany. The polyene antibiotics were generously donated by the following companies: The Squibb Institute for Medical Research, New Brunswick, N.J. (nystatin, amphotericin B); Farmitalia, Milan, Italy (etruscomycin); the Upjohn Company, Kalamazoo, Mich. (filipin and the filipin components); and Mycofarm-Delft, Division of Royal Netherlands Fermentation Industries Ltd., Delft, Holland (pimaricin).

The purity of the antibiotics was 90% or higher (information supplied by manufacturer). Molecular weights employed in the calculations were: filipin, 654; pimaricin, 681; etruscomycin, 700; nystatin, 932 and amphotericin B, 960.

Perhydrofilipin which lacks the conjugated double bond system, and irradiated filipin were prepared as described in an earlier report[25]. Because the product(s) of irradiated filipin have not been identified, a molecular weight of 654 was assumed. Stock solutions of the antibiotics were made with dimethyl formamide–water (57:100, v/v).

The bilayer membranes were formed across a 1-mm aperture in a Teflon septum separating two chambers containing 0.1 M NaCl (ref. 26). The films were generated from solutions of lipid in decane of the following composition: (a) 1% egg lecithin, (b) 1% egg lecithin and 0.5% cholesterol, (c) 1% egg lecithin and 0.05% cholesterol. Assuming a mol. wt. of 800 for egg lecithin, the molar ratio of lecithin:cholesterol in solutions (b) and (c) was 1:1 and 10:1, respectively.

In some experiments, egg lecithin was replaced by the synthetic (1,2-dioleoyl)-3-lecithin. The experiments with low antibiotic concentrations were initiated by the introduction of 0.01 ml antibiotic solution to one of the chambers of the cell. For the experiments with high antibiotic concentrations ($>10^{-5}$ M), the bilayer cell was filled with 0.1 M NaCl solution, which already contained the antibiotic before the film was generated. In experiments in which the influence of salts upon the antibiotic action was determined, the introduction of antibiotic was followed by the addition of 0.05 ml of the respective salt solution to the chamber containing the antibiotic or to the other chamber. In all cases, equal levels were maintained in both chambers so as to prevent rupture of the bilayer films. The electrical resistance of the bilayer films was measured with an electrometer (Keithley, 610 B), using reversible Ag/AgCl electrodes, which introduced an E of < 1 mV into the circuit. Membrane potentials were determined in the presence of a 10-fold concentration gradient between the two

chambers. For these measurements, use was made of calomel–KCl electrodes (Pye-Unican).

The salts used ($CaCl_2 \cdot 2H_2O$, $BaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $La(NO_3)_3$, NaCl, Na_2SO_4, Na_2HPO_4 and $NaNO_3$) were analytical grade. Ionic transference numbers (T_{ion}) were calculated from the membrane potential and the activity ratio of the ions in both chambers[27]. Activity coefficients were obtained from LATIMER[28] and PARSONS[29].

All bilayer experiments were carried out at 25° and the pH of the solutions was 5.8. The data presented are the mean values of at least five experiments.

RESULTS

Effects of polyenes on the stability and electrical resistance of lipid bilayers

Table I* demonstrates that films generated from an equimolar solution of lecithin and cholesterol were disrupted within 1 min after introduction of $4.0 \cdot 10^{-5}$ M filipin complex. Filipin II ($6.25 \cdot 10^{-6}$ M), filipin III ($6.25 \cdot 10^{-5}$ M), nystatin ($4.0 \cdot 10^{-5}$ M), etruscomycin ($4.0 \cdot 10^{-5}$ M), and pimaricin ($4.0 \cdot 10^{-5}$ M) disrupted these films after 4, 2, 1.5, 7 and 30 min, respectively.

Filipin complex appeared to be more lytic towards the bilayer membranes than nystatin, etruscomycin, and pimaricin. Thus, higher concentrations of nystatin, known to be less lytic than filipin complex towards both mammalian erythrocytes and Neurospora protoplasts[32], were required to disrupt the lecithin–cholesterol bilayer membranes in a given time. Even higher concentrations of etruscomycin and pimaricin, both polyenes of intermediate potency, were needed. This difference compared to their biological action can be explained by the poor solubility of these antibiotics.

When the activities of filipin complex and the filipin components are compared, it is obvious that filipin II is the most active in disrupting lecithin–cholesterol bilayer membranes, while filipin III and filipin complex are equal in this respect. Perhydrofilipin, which has approx. $^1/_{100}$–$^1/_{500}$ the potency of the parent antibiotic in hemolysis tests, and irradiated filipin, which has no detectable hemolytic activity, had no effect on the stability of bilayer membranes prepared from a lecithin–cholesterol 1:1 molar solution, at least at concn. of $4.0 \cdot 10^{-5}$ M. However, higher concentrations of perhydrofilipin and irradiated filipin (10^{-3} M) disrupted lecithin–cholesterol bilayer films rapidly.

Furthermore, in Table I it is demonstrated that the d.c. electrical resistance of the films generated from lecithin–cholesterol 1:1 molar solutions is reduced by a factor of 10 when treated with filipin complex or amphotericin B. A more pronounced effect on the d.c. resistance of lecithin–cholesterol bilayers was obtained with filipin II and filipin III, at concentrations of $6.25 \cdot 10^{-6}$–$6.25 \cdot 10^{-5}$ M. At these concentrations the survival times of the bilayer films were considerably reduced. Although all the other polyenes tested affected the permeability of biological membranes and the stability of lipid bilayers, no appreciable reduction of the bilayer resistance could be detected for any of them.

Addition of nystatin to one side of the bilayer film hardly affected its d.c. resistance, while addition to both sides lowered it by a factor of 10^3. On the other

* It should be noted that the data given in the earlier publications[15,16] were obtained without stirring the contents of the cell. This gives rise to longer survival times than those presented in the present paper.

TABLE I

THE EFFECT OF POLYENE ANTIBIOTICS ON THE STABILITY AND THE ELECTRICAL RESISTANCE OF EGG LECITHIN–CHOLESTEROL 1:1 BILAYER MEMBRANES, GENERATED IN 0.1 M NaCl SOLUTIONS

The membrane resistances are expressed as the mean ± S.D. Values in parentheses indicate the range.

Addition	*Concentration (M)*	*Average survival time of film (min)*	*Membrane resistance ($\Omega \cdot cm^2$)*
None	—	>60	$7.0 \pm 1.0 \cdot 10^7$
Filipin complex	$4.0 \cdot 10^{-7}$	>60	$4.3 \pm 0.9 \cdot 10^6$
	$4.0 \cdot 10^{-6}$	6 (3–10)	$4.0 \pm 1.1 \cdot 10^6$
	$4.0 \cdot 10^{-5}$	< 1	
Filipin II	$6.25 \cdot 10^{-8}$	>60	$1.6 \pm 0.9 \cdot 10^6$
	$6.25 \cdot 10^{-7}$	10 (8–11)	$2.0 \pm 1.0 \cdot 10^5$
	$6.25 \cdot 10^{-6}$	4 (3–6)	$4.7 \pm 0.7 \cdot 10^4$
Filipin III	$4.0 \cdot 10^{-7}$	>60	$4.0 \pm 1.5 \cdot 10^6$
	$6.25 \cdot 10^{-6}$	5 (4–8)	$2.0 \pm 0.5 \cdot 10^6$
	$6.25 \cdot 10^{-5}$	2 (1.5–3)	$5.0 \pm 0.6 \cdot 10^4$
Nystatin	$4.0 \cdot 10^{-7}$	>60	$5.6 \pm 1.3 \cdot 10^7$
	$4.0 \cdot 10^{-6}$	23 (15–27)	$2.9 \pm 1.1 \cdot 10^7$
	$4.0 \cdot 10^{-5}$	1.5 (1–2)	$2.7 \pm 1.2 \cdot 10^7$
Nystatin *	$4.0 \cdot 10^{-7}$	>60	$2.5 \pm 0.5 \cdot 10^5$
	$6.25 \cdot 10^{-6}$	2 (1.5–3)	$5.0 \pm 0.4 \cdot 10^4$
Etruscomycin	$4.0 \cdot 10^{-7}$	>60	
	$2.0 \cdot 10^{-5}$	12 (8–20)	
	$4.0 \cdot 10^{-5}$	7 (6–12)	$3.0 \pm 2.0 \cdot 10^7$
Pimaricin	$4.0 \cdot 10^{-5}$	30 (20–45)	$3.5 \pm 0.7 \cdot 10^7$
Amphotericin B *	$4.0 \cdot 10^{-7}$	>60	$5.0 \pm 1.1 \cdot 10^6$
Perhydrofilipin	$4.0 \cdot 10^{-5}$	>60	$3.2 \pm 1.1 \cdot 10^7$
	$5.0 \cdot 10^{-3}$	5 (3.5–7)	
Irradiated filipin	$4.0 \cdot 10^{-5}$	>60	$2.5 \pm 0.4 \cdot 10^7$
	$8.0 \cdot 10^{-5}$	>60	$2.1 \pm 0.6 \cdot 10^7$
	$1.0 \cdot 10^{-3}$	4 (3–6)	

* Added to both sides.

hand, addition of amphotericin B to one side of the film reduced its d.c. resistance by a factor of 10, while addition to both sides gave inconclusive results, *e.g.* $4.0 \cdot 10^{-7}$ M amphotericin B, added to both sides of a given membrane might lower its d.c. resistance by a factor of 10^4, whereas the same concentration acting on another film generated from the same film-forming solution might have no effect at all (compare Table I and refs. 17, 18).

Table II compares the effects of $4.0 \cdot 10^{-6}$ M filipin complex and $4.0 \cdot 10^{-6}$ M nystatin on the stability and the electrical resistance of lipid bilayers when these were generated from solutions with various molar ratios of egg lecithin–cholesterol. The survival times of bilayer membranes generated from lecithin–cholesterol 10:1

TABLE II

THE EFFECT OF $4.0 \cdot 10^{-6}$ M FILIPIN COMPLEX AND $4.0 \cdot 10^{-6}$ M NYSTATIN ON THE STABILITY AND THE ELECTRICAL RESISTANCE OF LIPID BILAYERS, GENERATED FROM SOLUTIONS WITH VARIOUS MOLAR RATIOS OF EGG LECITHIN–CHOLESTEROL

The aqueous phase contained 0.1 M NaCl. The membrane resistances are expressed as the mean ± S.D. Values in parentheses indicate the range.

Composition of film-forming solution	*Addition*	*Average survival time of film (min)*	*Membrane resistance* ($\Omega \cdot cm^2$)
Lecithin–cholesterol 10:1 molar	None	>60	$6.0 \pm 0.6 \cdot 10^6$
	Filipin complex	20 – >60**	$4.4 \pm 0.8 \cdot 10^6$
	Nystatin	30 – >60**	$4.5 \pm 0.5 \cdot 10^6$
Lecithin–cholesterol 1:1 molar	None	>60	$7.0 \pm 1.0 \cdot 10^7$
	Filipin complex	6 (3–10)	$4.0 \pm 1.1 \cdot 10^6$
	Nystatin	23 (15–27)	$2.9 \pm 1.1 \cdot 10^7$
	Nystatin*	10 (8–13)	$5.6 \pm 0.5 \cdot 10^4$
Lecithin–cholesterol 1:2 molar	None	>60	$3.0 \pm 1.0 \cdot 10^7$
	Filipin complex	15 (12–18)	$1.2 \pm 1.0 \cdot 10^6$
Lecithin–cholesterol 1:3 molar	None	>60	$8.1 \pm 2.1 \cdot 10^7$
	Filipin complex	13 (11–16)	$7.0 \pm 2.0 \cdot 10^6$
	Nystatin	19 (16–23)	$3.5 \pm 0.5 \cdot 10^7$
	Nystatin*	9 (8–12)	$3.0 \pm 1.0 \cdot 10^4$

* Added to both sides.
** See text for discussion.

and 1:1 molar solutions, show that an increase in the amount of cholesterol in the film-forming solution decreases the stability of the bilayers towards filipin complex and nystatin. A further increase in the amount of cholesterol (bilayer membrane generated from lecithin–cholesterol 1:2 and 1:3 molar solutions) increases the survival times. This last effect may be caused by complex formation between antibiotic and cholesterol, not present in the black film itself, but in its meniscus, thus decreasing the effective polyene concentration. It has already been demonstrated for filipin complex[15] that some of the bilayers generated from a lipid solution with a 10:1 molar ratio of lecithin–cholesterol remained stable for at least 1 h in the presence of $4.0 \cdot 10^{-6}$ M antibiotic. However, other films, formed from a solution with the same lipid composition had an average survival time of 20 min, when filipin complex was added at this concentration. Experiments with nystatin carried out under identical conditions, showed the same difference in survival times. The reason for this difference is not yet known, but it may reflect a varying lipid composition of the bilayers. In addition, it must be mentioned that lipid bilayer membranes formed from lecithin–cholesterol 10:1 molar solutions had sharp survival times, when filipin complex or nystatin were added at higher or lower concentrations ($4.0 \cdot 10^{-5}$ and $4.0 \cdot 10^{-7}$ M).

Table II shows that the resistance of bilayer films generated from lecithin–cholesterol 10:1 molar solutions was not affected by $4.0 \cdot 10^{-6}$ M filipin complex. However, the resistance of bilayer membranes, generated from lecithin–cholesterol 1:1, 1:2, and 1:3 molar solutions was reduced by a factor of 10 in the presence of this concentration of filipin complex (compare Table I). Nystatin, added to one side

TABLE III

THE EFFECT OF POLYENE ANTIBIOTICS ON THE STABILITY AND THE ELECTRICAL RESISTANCE OF EGG LECITHIN BILAYER MEMBRANES, GENERATED IN 0.1 M NaCl SOLUTIONS

The membrane resistances are expressed as the mean ± S.D. Values in parentheses indicate the range.

Addition	*Concentration (M)*	*Average survival time of film (min)*	*Membrane resistance ($\Omega \cdot cm^2$)*
None	—	>60	$7.5 \pm 0.8 \cdot 10^6$
Filipin complex	$4.0 \cdot 10^{-7}$	>60	$3.9 \pm 1.1 \cdot 10^6$
	$4.0 \cdot 10^{-5}$	>60	$4.5 \pm 0.5 \cdot 10^6$
	$1.0 \cdot 10^{-4}$	7.5 (3.5–10)	$3.7 \pm 0.5 \cdot 10^6$
Filipin II	$6.25 \cdot 10^{-7}$	>60	$4.9 \pm 0.6 \cdot 10^6$
	$6.25 \cdot 10^{-5}$	>60	$4.0 \pm 0.5 \cdot 10^6$
Filipin III	$6.25 \cdot 10^{-7}$	>60	$5.0 \pm 1.0 \cdot 10^6$
	$6.25 \cdot 10^{-5}$	>60	$4.3 \pm 0.4 \cdot 10^6$
Nystatin	$4.0 \cdot 10^{-5}$	>60	$7.0 \pm 1.0 \cdot 10^6$
	$1.0 \cdot 10^{-4}$	>60	
	$8.0 \cdot 10^{-3}$	32.5 (15–54)	
	$1.0 \cdot 10^{-2}$	8 (6–10)	
Etruscomycin	$4.0 \cdot 10^{-5}$	>60	$5.2 \pm 1.5 \cdot 10^6$
Pimaricin	$4.0 \cdot 10^{-5}$	>60	$4.1 \pm 1.0 \cdot 10^6$
Amphotericin B	$4.0 \cdot 10^{-7}$	>60	$4.7 \pm 1.7 \cdot 10^6$
Perhydrofilipin	$4.0 \cdot 10^{-5}$	>60	
	$5.0 \cdot 10^{-3}$	5.5 (4–10)	
Irradiated filipin	$4.0 \cdot 10^{-5}$	>60	
	$1.0 \cdot 10^{-3}$	1 (0.5–1.5)	

of the lecithin–cholesterol bilayer films had no effect at all on their electrical resistances, but reduced the film resistance by a factor of 10^3 when added to both sides.

As shown in Table III, bilayer membranes containing only lecithin were stable in the presence of filipin complex ($4.0 \cdot 10^{-5}$ M) or the filipin components II and III, at concentrations of $6.25 \cdot 10^{-5}$ M. They were also stable in the presence of nystatin ($1.0 \cdot 10^{-4}$ M), etruscomycin ($4.0 \cdot 10^{-5}$ M), pimaricin ($4.0 \cdot 10^{-5}$ M), and amphotericin B ($4.0 \cdot 10^{-7}$ M). Higher concentrations of amphotericin B caused precipitation of the antibiotic in the bilayer cell. The survival times of lecithin bilayer membranes were considerably reduced when filipin complex or nystatin was added to both chambers of the bilayer cell, at concentrations of $1.0 \cdot 10^{-4}$ or $8.0 \cdot 10^{-3}$ M, respectively. The poor solubility of etruscomycin and pimaricin made it impossible to determine whether lecithin bilayers are disrupted at antibiotic concentrations of $>4.0 \cdot 10^{-5}$ M. The filipin derivatives, perhydrofilipin and irradiated filipin, at concentrations of $4.0 \cdot 10^{-5}$ M, had no effect on the stability of lecithin and lecithin–cholesterol bilayer membranes (compare Table I), which is consistent with the biological activities of these derivatives. Concentrations of 10^{-3} M perhydrofilipin and irradiated filipin did disrupt

bimolecular lecithin films rapidly. Furthermore, in Table III, it is demonstrated that lecithin bilayer films which are not affected by low concentrations of polyene antibiotics do not show a significant reduction of the d.c. electrical resistances. Even with high concentrations of filipin complex, which disrupt the lecithin films, the bilayer resistance was hardly changed within the survival time.

The results given in Tables I, II and III are based on experiments in which the antibiotics were added either to one or to both chambers of the bilayer cell. Table IV compares the results of experiments in which the antibiotics (filipin complex,

TABLE IV

THE EFFECT OF FILIPIN COMPLEX, SOME FILIPIN COMPONENTS, AND NYSTATIN, ON THE STABILITY AND THE ELECTRICAL RESISTANCE OF EGG LECITHIN–CHOLESTEROL BILAYER MEMBRANES

The antibiotics were present in the film-forming solution. The aqueous phase contained 0.1 M NaCl. The membrane resistances are expressed as the mean ± S.D.

Lipids in film-forming solution	*Antibiotic in film-forming solution*	*Molar ratio antibiotic/lipid*	*Average survival time of film (min)*	*Membrane resistance* ($\Omega \cdot cm^2$)
Lecithin–cholesterol 1:1 molar	—	—	>60	$7.0 \pm 1.0 \cdot 10^7$
	Filipin complex	16:200	>60	$5.2 \pm 0.7 \cdot 10^4$
	Filipin II	16:200	>60	$8.0 \pm 1.6 \cdot 10^5$
	Filipin III	16:200	>60	$4.0 \pm 0.5 \cdot 10^4$
	Nystatin	43:200	>60	$7.5 \pm 1.4 \cdot 10^5$

filipin II, filipin III, and nystatin) were added to the film-forming solution. Only these antibiotics gave stable film-forming solutions, while the other polyenes tested (pimaricin, etruscomycin, and amphotericin B) did not dissolve at all in the lipid–decane system. The molar ratios of antibiotic/lipid given in Table IV were the highest that could be used, since larger proportions of antibiotics in the film-forming solution led to unstable bilayers. Although it could be assumed that a rather large number of lytic antibiotic molecules were present in the bilayer films, every film remained stable for more than 1 h. The bilayer membranes containing filipin complex or filipin III showed d.c. resistance of about $5 \cdot 10^4\ \Omega \cdot cm^2$. The films containing filipin II or nystatin had resistances of about $8 \cdot 10^5\ \Omega \cdot cm^2$. However, to obtain equally-conducting bilayer membranes, the amount of nystatin in the film-forming solution had to be appreciably higher than the amount of filipin II. If we compare the d.c. resistance of lecithin–cholesterol 1:1 bilayer films with those of membranes containing filipin complex or its components, it is clear, that filipin complex and filipin III lower the resistance to a greater extent than filipin II (compare the results of Table I).

Effects of polyenes on the electrical resistance of lipid bilayers in the presence of several ions

The results compiled in Figs. 1–4 compare the influence of several cations and anions upon the d.c. resistance of lipid bilayer membranes, when treated or untreated with polyene antibiotics. As stated before, NORMAN[20,41] found that filipin complex was capable of increasing the Ca^{2+} permeability of intestinal mucosal cells obtained from rachitic chicks. Our experiments were carried out to investigate whether or not

filipin complex and other polyene antibiotics were able to increase the ion permeability of lipid bilayer membranes in a specific manner.

The bilayer films were formed in 0.1 M NaCl from solutions which contained synthetic (1,2-dioleoyl)-3-lecithin as phospholipid (see MATERIALS AND METHODS). The antibiotics were introduced to one chamber of the bilayer cell, followed by the

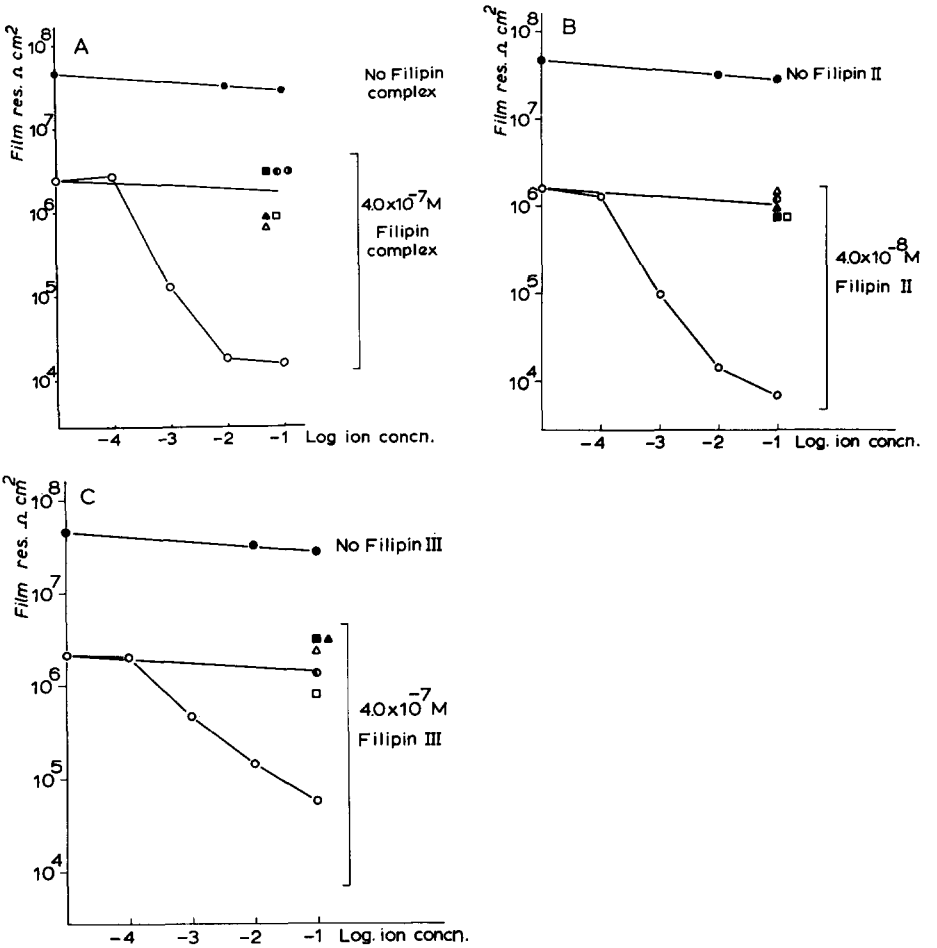

Fig. 1. A. The effect of filipin complex and various salt solutions on the d.c. resistance of lipid bilayer membranes. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. $4.0 \cdot 10^{-7}$ M filipin complex and $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)_3$ (△), $MgCl_2$ (■), Na_2HPO_4 (□), $NaNO_3$ (◐), or $BaCl_2$ (◑), were added to one compartment. The d.c. resistance of the films when $CaCl_2$ but no filipin complex was added is denoted by ●. B. The effect of filipin II and various salt solutions on the d.c. resistance of lipid bilayer membranes. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. $4.0 \cdot 10^{-8}$ M filipin II and $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)_3$ (△), $MgCl_2$ (■), Na_2HPO_4 (□), or $NaNO_3$ (◐) were added to one compartment. The d.c. resistance of the films when $CaCl_2$ but no filipin II was added is denoted by ●. C. The effect of filipin III and various salt solutions on the d.c. resistance of lipid bilayer membranes. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. $4.0 \cdot 10^{-7}$ M filipin III and $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)_3$ (△), $MgCl_2$ (■), Na_2HPO_4 (□), or $NaNO_3$ (◐) were added to one compartment. The d.c. resistance of the films when $CaCl_2$ but no filipin III was added is denoted by ●.

addition of the appropriate salt solution to the same chamber or to the other one.

Fig. 1A shows that the addition of Ca^{2+} up to a concentration of 0.1 M had no effect on the d.c. resistance of lipid bilayers formed from lecithin–cholesterol 1:1 molar solutions. This result is in agreement with the findings af PAPAHADJOPOULOS AND OHKI[33]. The addition of $4.0 \cdot 10^{-7}$ M filipin complex to one side of these films reduced their resistance by a factor of 10 (compare Table I). Subsequent addition of Mg^{2+}, Ba^{2+}, La^{3+}, SO_4^{2-}, HPO_4^{2-}, and NO_3^- had no effect on the bilayer resistance. However, subsequent addition of Ca^{2+} up to a concentration of 0.1 M lowered the resistance of the lecithin–cholesterol bilayers by another factor of 10^2. It should be mentioned that the final bilayer resistance was not influenced by the order of introduction of filipin complex and Ca^{2+}. Films prepared from (1,2-dioleoyl)-3-lecithin only did not show any decrease in resistance after addition of filipin complex at a concentration of $4.0 \cdot 10^{-7}$ M and subsequent introduction of Ca^{2+}, or of the other ions mentioned.

Figs. 1B and 1C demonstrate that the data obtained for $4.0 \cdot 10^{-8}$ M filipin II and $4.0 \cdot 10^{-7}$ M filipin III were comparable with those for filipin complex. Although the concentration of filipin II was 10 times lower than that of filipin III or filipin complex, its combination with 0.1 M Ca^{2+} gave rise to lower bilayer resistances than the combinations of either filipin III or filipin complex and Ca^{2+}.

Figs. 2A and 2B compare the results of the same type of experiments carried out with nystatin on one side and on both sides of lecithin–cholesterol bilayer films. It is demonstrated (Fig. 2A) that addition of $4.0 \cdot 10^{-7}$ M nystatin to one side of these bilayers followed by the addition of Ca^{2+} up to a concentration of 0.1 M reduced the

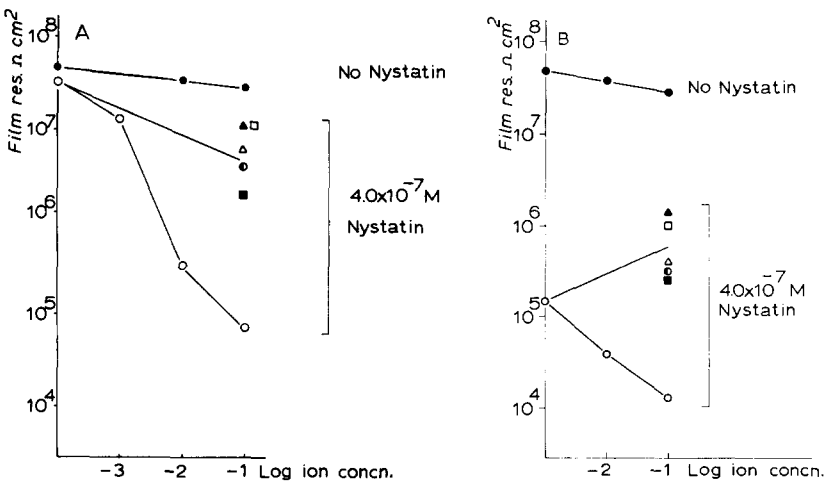

Fig. 2. A. The effect of nystatin and various salt solutions on the d.c. resistance of lipid bilayer membranes. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. $4.0 \cdot 10^{-7}$ M nystatin and $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)_3$ (△), $MgCl_2$ (■), Na_2HPO_4 (□), or $NaNO_3$ (◐) were added to one compartment. The d.c. resistance of the films when $CaCl_2$ but no nystatin was added is denoted by ●. B. The effect of nystatin on the d.c. resistance of lipid bilayer membranes when added to both compartments. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. Both compartments contained $4.0 \cdot 10^{-7}$ M nystatin. $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)_3$ (△), $MgCl_2$ (■), Na_2HPO_4 (□), or $NaNO_3$ (◐) were added to one compartment. The d.c. resistance of the films when $CaCl_2$ but no nystatin was added is denoted by ●.

resistances by a factor of $5 \cdot 10^2$ (compare Table I). Introduction of $4.0 \cdot 10^{-7}$ M nystatin to both sides of the lecithin–cholesterol 1:1 bilayer membranes (Fig. 2B) lowered the resistance by a factor of $3 \cdot 10^2$, while subsequent addition of Ca^{2+} up to a concentration of 0.1 M reduced them by a further factor of 10.

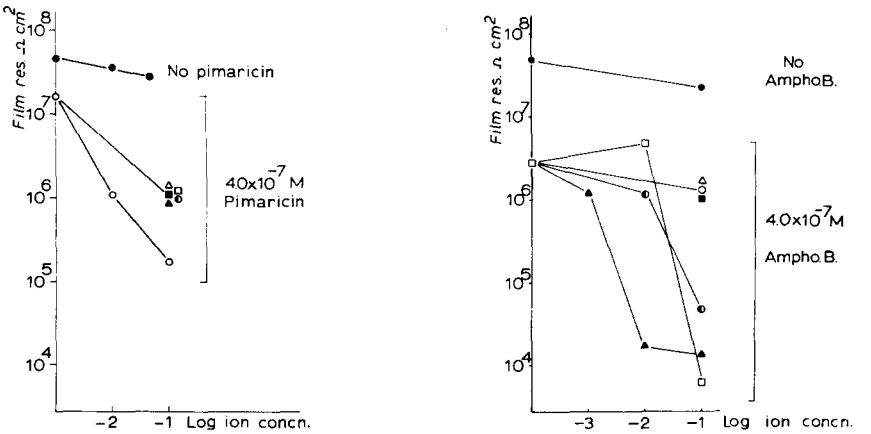

Fig. 3. The effect of pimaricin and various salt solutions on the d.c. resistance of lipid bilayer membranes. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. $4.0 \cdot 10^{-7}$ M pimaricin and $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)_3$ (△), $MgCl_2$ (■), Na_2HPO_4 (□), or $NaNO_3$ (◐) were added to one compartment. The d.c. resistance of the films when $CaCl_2$ but no pimaricin was added is denoted by ●.

Fig. 4. The effect of amphotericin B and various salt solutions on the d.c. resistance of lipid bilayer membranes. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions in 0.1 M NaCl. $4.0 \cdot 10^{-7}$ M amphotericin B and $CaCl_2$ (○), Na_2SO_4 (▲), $La(NO_3)$ (△), $MgCl_2$ (■), $NaHPO_4$ (□), or $NaNO_3$ (◐) were added to one compartment. The d.c. resistance of the films when either $CaCl_2$, $NaNO_3$, $NaSO_4$, or Na_2HPO_4 but no amphotericin B was added is denoted by ●.

Fig. 3 shows that the d.c. resistance of lecithin–cholesterol 1:1 bilayer membranes was lowered with pimaricin, especially in the presence of Ca^{2+}, whereas other cations and anions had a less pronounced effect. On the other hand, amphotericin B, which is known to be a positively charged polyene antibiotic[34] caused a decrease in the d.c. resistance of lecithin–cholesterol 1:1 bilayer films in the presence of anions such as HPO_4^{2-}, NO_3^-, and SO_4^{2-} (Fig. 4). The addition of several cations produced only a very limited effect. It should be noted that the d.c. resistance of bilayer membranes prepared from lecithin only, was not affected by the presence of the various ions and polyene antibiotics.

Effects of polyenes on the ion selectivity of lipid bilayers

Tables V–XI give the results of experiments in which the effects of polyene antibiotics on the d.c. resistance of lipid bilayers formed in different salt solutions were determined. These tables also demonstrate the effects of polyenes on the ion selectivity of lipid bilayer films.

As shown in Table V, in the absence of filipin complex, lecithin and lecithin–cholesterol bilayer membranes were slightly cation selective in the presence of monovalent cations and anion selective in the presence of di- and trivalent cations.

TABLE V

THE EFFECT OF $4.0 \cdot 10^{-7}$ M FILIPIN COMPLEX ON THE ION SELECTIVITY OF LIPID BILAYER MEMBRANES

The membrane resistances are expressed as the mean ± S.D. When the resistances were measured, the salt concentration in both chambers was 0.1 M. The membrane potentials were measured when the chambers contained the salt concentrations indicated in the table. The range of the observations is given by the values in parentheses. The transference numbers were calculated from the membrane potentials, as indicated in MATERIALS AND METHODS.

Composition of film-forming solution	*Aqueous phase*		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
	Rear chamber (0.1 *M*)	*Front chamber* (0.01 *M*)				$T+$	$T-$
(1,2-Dioleoyl)-3-lecithin	NaCl	NaCl	—	$8.0 \pm 1.0 \cdot 10^6$	− 6.9 (5–9)	0.56	0.44
			+	$4.8 \pm 0.9 \cdot 10^6$	− 7.8 (5–11)	0.57	0.43
(1,2-Dioleoyl)-3-lecithin/cholesterol 1:1 molar	NaCl	NaCl	—	$7.0 \pm 0.5 \cdot 10^7$	− 5.3 (3–7)	0.55	0.45
			+	$4.5 \pm 0.5 \cdot 10^6$	−17.5 (14–21)	0.66	0.34
	$CaCl_2$	$CaCl_2$	—	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
			+	$2.0 \pm 1.0 \cdot 10^4$	−11.7 (9–15)	0.82	0.09
	$MgCl_2$	$MgCl_2$	—	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
			+	$3.9 \pm 0.4 \cdot 10^6$	+26.7 (25–29)	0.32	0.34
	$La(NO_3)_3$	$La(NO_3)_3$	—	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
			+	$6.0 \pm 2.0 \cdot 10^6$	+50.4 (46–52)	0.28	0.24
	$NaNO_3$	$NaNO_3$	—	$8.0 \pm 1.5 \cdot 10^7$	− 7.1 (4–10)	0.56	0.44
			+	$5.2 \pm 0.4 \cdot 10^6$	−19.6 (15–24)	0.68	0.32

TABLE VI

THE EFFECT OF $4.0 \cdot 10^{-8}$ M FILIPIN II ON THE ION SELECTIVITY OF (1,2-DIOLEOYL)-3-LECITHIN/CHOLESTEROL BILAYER MEMBRANES

The conditions in this table are the same as in Table V.

Aqueous phase		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
Rear chamber (0.1 *M*)	*Front chamber* (0.01 *M*)				$T+$	$T-$
NaCl	NaCl	—	$7.0 \pm 0.5 \cdot 10^7$	− 5.3 (3–7)	0.55	0.45
		+	$2.8 \pm 0.8 \cdot 10^6$	−18.4 (15–21)	0.67	0.33
$CaCl_2$	$CaCl_2$	—	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
		+	$9.5 \pm 0.5 \cdot 10^3$	−15.9 (12–20)	0.88	0.06
$MgCl_2$	$MgCl_2$	—	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
		+	$3.5 \pm 1.4 \cdot 10^6$	+28.7 (25–31)	0.30	0.35
$La(NO_3)_3$	$La(NO_3)_3$	—	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
		+	$4.0 \pm 2.2 \cdot 10^6$	+53.4 (51–56)	0.22	0.26
$NaNO_3$	$NaNO_3$	—	$8.0 \pm 1.5 \cdot 10^7$	− 7.1 (4–10)	0.56	0.44
		+	$3.0 \pm 1.1 \cdot 10^6$	−19.6 (15–22)	0.68	0.32

When lecithin bilayers were formed in 0.1 M NaCl solutions, addition of $4.0 \cdot 10^{-7}$ M filipin complex neither affected the d.c. resistance of these membranes (compare Table III), nor the ionic transference numbers T_{Na^+} and T_{Cl^-}. If lecithin–cholesterol bilayers were formed in either NaCl, $MgCl_2$, $La(NO_3)_3$ or $NaNO_3$, addition of $4.0 \cdot 10^{-7}$ M filipin complex decreased their resistances by a factor of about 10 *pari pasu* with a reasonable increase in T_{cation}. However, the combination of $4.0 \cdot 10^{-7}$ M

TABLE VII

THE EFFECT OF $4.0 \cdot 10^{-7}$ M FILIPIN III ON THE ION SELECTIVITY OF (1,2-DIOLEOYL)-3-LECITHIN/CHOLESTEROL BILAYER MEMBRANES

The conditions in this table are the same as in Table V.

Aqueous phase		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
Rear chamber (2.1 *M*)	*Front chamber* (0.01 *M*)				$T+$	$T-$
NaCl	NaCl	–	$7.0 \pm 0.5 \cdot 10^7$	− 5.3 (3–7)	0.55	0.45
		+	$4.0 \pm 1.5 \cdot 10^6$	−16.4 (14–20)	0.65	0.35
$CaCl_2$	$CaCl_2$	–	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
		+	$8.0 \pm 1.5 \cdot 10^4$	− 8.6 (6–11)	0.78	0.11
$MgCl_2$	$MgCl_2$	–	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
		+	$4.0 \pm 2.0 \cdot 10^6$	+31.4 (28–35)	0.26	0.37
$La(NO_3)_3$	$La(NO_3)_3$	–	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
		+	$4.5 \pm 2.0 \cdot 10^6$	+55.2 (54–60)	0.22	0.26
$NaNO_3$	$NaNO_3$	–	$8.0 \pm 1.5 \cdot 10^7$	− 7.1 (4–10)	0.56	0.44
		+	$2.0 \pm 0.5 \cdot 10^6$	−18.7 (13–20)	0.67	0.33

TABLE VIII

THE EFFECT OF $4.0 \cdot 10^{-7}$ M NYSTATIN ON THE ION SELECTIVITY OF (1,2-DIOLEOYL)-3-LECITHIN/CHOLESTEROL BILAYER MEMBRANES

Nystatin was added to one chamber only. The conditions in this table are the same as in Table V.

Aqueous phase		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
Rear chamber (0.1 *M*)	*Front chamber* (0.01 *M*)				$T+$	$T-$
NaCl	NaCl	–	$7.0 \pm 0.5 \cdot 10^7$	− 5.3 (3–7)	0.55	0.45
		+	$5.6 \pm 1.3 \cdot 10^7$	− 5.4 (4–7)	0.55	0.45
$CaCl_2$	$CaCl_2$	–	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
		+	$9.0 \pm 1.4 \cdot 10^7$	+12.2 (9–15)	0.50	0.25
$MgCl_2$	$MgCl_2$	–	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
		+	$6.0 \pm 1.5 \cdot 10^6$	+36.3 (35–40)	0.20	0.40
$La(NO_3)_3$	$La(NO_3)_3$	–	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
		+	$9.5 \pm 0.5 \cdot 10^6$	+62.4 (57–64)	0.16	0.28
$NaNO_3$	$NaNO_3$	–	$8.0 \pm 1.5 \cdot 10^7$	− 7.1 (4–10)	0.56	0.44
		+	$7.5 \pm 1.5 \cdot 10^6$	−11.5 (7–16)	0.61	0.39

filipin complex and $CaCl_2$ in the aqueous phase, yielded highly conducting, highly Ca^{2+}-selective lecithin–cholesterol bilayer membranes. Although $T_{Ca^{2+}}$ did not become unity, it was approx. 10 times as great as T_{Cl^-}.

Tables VI–VIII and X demonstrate that the results obtained for filipin II, filipin III, nystatin (when added to one side of the bilayer films) and pimaricin were similar to those obtained for filipin complex. It is obvious that filipin II was more

TABLE IX

THE EFFECT OF $4.0 \cdot 10^{-7}$ M NYSTATIN ON THE ION SELECTIVITY OF (1,2-DIOLEOYL)-3-LECITHIN/CHOLESTEROL BILAYER MEMBRANES

Nystatin was added to both chambers. The conditions in this table are the same as in Table V.

Aqueous phase		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
Rear chamber (*0.1 M*)	*Front chamber* (*0.01 M*)				$T+$	$T-$
NaCl	NaCl	−	$7.0 \pm 0.5 \cdot 10^7$	− 5.3 (3–7)	0.55	0.45
		+	$2.5 \pm 1.5 \cdot 10^5$	+45.4 (43–48)	0.09	0.91
$CaCl_2$	$CaCl_2$	−	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
		+	$2.0 \pm 0.4 \cdot 10^4$	− 6.9 (5–11)	0.76	0.12
$MgCl_2$	$MgCl_2$	−	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
		+	$5.5 \pm 1.5 \cdot 10^5$	+20.6 (18–22)	0.40	0.30
$La(NO_3)_3$	$La(NO_3)_3$	−	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
		+	$6.5 \pm 0.5 \cdot 10^5$	+56.5 (45–62)	0.22	0.26
$NaNO_3$	$NaNO_3$	−	$8.0 \pm 1.5 \cdot 10^7$	− 7.1 (4–10)	0.56	0.44
		+	$6.0 \pm 1.5 \cdot 10^5$	+44.4 (42–48)	0.10	0.90

TABLE X

THE EFFECT OF $4.0 \cdot 10^{-7}$ M PIMARICIN ON THE ION SELECTIVITY OF (1,2-DIOLEOYL)-3-LECITHIN/CHOLESTEROL BILAYER MEMBRANES

The conditions in this table are the same as in Table V.

Aqueous phase		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
Rear chamber (*0.1 M*)	*Front chamber* (*0.01 M*)				$T+$	$T-$
NaCl	NaCl	−	$7.0 \pm 0.5 \cdot 10^7$	− 5.3 (3–7)	0.55	0.45
		+	$2.8 \pm 1.3 \cdot 10^7$	− 7.6 (6–10)	0.57	0.43
$CaCl_2$	$CaCl_2$	−	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
		+	$2.0 \pm 0.6 \cdot 10^5$	+14.2 (12–18)	0.48	0.26
$MgCl_2$	$MgCl_2$	−	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
		+	$1.0 \pm 0.5 \cdot 10^6$	+33.0 (30–36)	0.24	0.38
$La(NO_3)_3$	$La(NO_3)_3$	−	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
		+	$1.5 \pm 0.5 \cdot 10^6$	+61.6 (56–65)	0.16	0.28
$NaNO_3$	$NaNO_3$	−	$8.0 \pm 1.5 \cdot 10^7$	− 7.1 (4–10)	0.56	0.44
		+	$2.5 \pm 1.0 \cdot 10^6$	−14.5 (10–16)	0.63	0.37

potent than the other antibiotics mentioned, although its final concentration was 10 times as low. Thus if lecithin–cholesterol bilayers were formed in $CaCl_2$, addition of $4.0 \cdot 10^{-8}$ M filipin II led to a definite reduction of their d.c. resistance, while $T_{Ca^{2+}}$ was approx. 15 times as great as T_{Cl^-}.

Table IX compiles the results for nystatin when this antibiotic was added to both sides of lecithin–cholesterol bilayer membranes. If membranes were formed in either NaCl or $NaNO_3$, addition of $4.0 \cdot 10^{-7}$ M nystatin to both sides reduced their resistances by a factor of 100 and rendered them highly anion-selective (both T_{Cl^-} and $T_{NO_3^-}$ were 10 times as great as T_{Na^+}). Nystatin produced an identical reduction of the d.c. resistance of lecithin–cholesterol membranes formed in either $MgCl_2$ or $La(NO_3)_3$, but the membranes became rather cation selective. Furthermore, it is clear from Table IX that the combination of nystatin (added to both sides of the films) and $CaCl_2$, produced highly conducting and highly Ca^{2+}-selective lecithin–cholesterol bilayers. Table XI shows that amphotericin B produced anion-selective lecithin–cholesterol bilayers but a pronounced effect could be observed only when $NaNO_3$ was present in the aqueous phase. Thus, the resistance of lecithin–cholesterol bilayers in $NaNO_3$ was lowered by a factor of 10^3 upon addition of $4.0 \cdot 10^{-7}$ M amphotericin B, while $T_{NO_3^-}$ was 3.5 times as great as T_{Na^+}.

TABLE XI

THE EFFECT OF $4.0 \cdot 10^{-7}$ M AMPHOTERICIN B ON THE ION SELECTIVITY OF (1,2-DIOLEOYL)-3-LECITHIN/CHOLESTEROL BILAYER MEMBRANES

The conditions in this table are the same as in Table V.

Aqueous phase		*Antibiotic*	*Membrane resistance* ($\Omega \cdot cm^2$)	*Membrane potential* (*mV*)	*Transference numbers*	
Rear chamber (*0.1 M*)	*Front chamber* (*0.01 M*)				$T+$	$T-$
NaCl	NaCl	–	$7.0 \pm 0.5 \cdot 10^7$	−5.3 (3–7)	0.55	0.45
		+	$5.0 \pm 2.0 \cdot 10^6$	+13.4 (10–16)	0.38	0.62
$CaCl_2$	$CaCl_2$	–	$7.6 \pm 0.8 \cdot 10^7$	+44.2 (39–47)	0.08	0.46
		+	$3.1 \pm 1.1 \cdot 10^6$	+65.0 (60–68)	0.04	0.48
$MgCl_2$	$MgCl_2$	–	$7.0 \pm 0.6 \cdot 10^7$	+43.8 (39–45)	0.10	0.45
		+	$2.0 \pm 0.5 \cdot 10^6$	+70.0 (65–74)	0.06	0.47
$La(NO_3)_3$	$La(NO_3)_3$	–	$7.9 \pm 0.6 \cdot 10^7$	+71.3 (65–72)	0.04	0.32
		+	$2.5 \pm 1.5 \cdot 10^6$	+80.0 (75–85)	0.01	0.33
$NaNO_3$	$NaNO_3$	–	$8.0 \pm 1.5 \cdot 10^7$	−7.1 (4–10)	0.56	0.44
		+	$8.0 \pm 1.2 \cdot 10^4$	+31.0 (27–36)	0.22	0.78

DISCUSSION

The results compiled in Tables I–IV show that bilayer membranes generated from lipid solutions containing lecithin and cholesterol in a 1:1 molar ratio are disrupted by polyene antibiotics at concentrations which, depending upon the potency of the antibiotics, range from $4.0 \cdot 10^{-6}$ to $4.0 \cdot 10^{-5}$ M. It appears that at these concentrations, the survival times of lecithin–cholesterol 1:1 bilayer membranes correspond fairly well with the physiological activities of the polyenes and their derivatives[7].

At much higher concentrations ($>10^{-4}$ M) filipin, its derivatives, and the other polyene antibiotics are able to disrupt bilayer membranes formed from solutions containing lecithin and cholesterol in a 10:1 molar ratio or lecithin alone. Our finding that these antibiotics are able to disrupt lecithin bilayer membranes, provided that high concentrations ($>10^{-4}$ M) are used, are in good agreement with those of DEMEL *et al.*[7] on monolayers and those of WEISSMANN AND SESSA[8], and KINSKY *et al.*[10] on liposomes. We have to consider the possibility that the disruption of bimolecular lipid membranes and liposomes observed with high antibiotic concentrations may not be due to a direct interaction with the artificial membrane systems at all, but to a surface tension-lowering effect of the amphipathic molecules. We cannot even exclude the fact that the latter also plays a role in the action of low concentrations of unmodified polyenes on membranes.

The d.c. resistances of lecithin bilayer membranes appear to be unaffected by the polyene antibiotics. In other words, the polyene antibiotics do not influence the ion permeability of lecithin bilayers. The d.c. resistances of bilayer membranes prepared from lecithin–cholesterol 1:1 molar solutions show no reduction after addition of nystatin, etruscomycin, pimaricin, perhydrofilipin, and irradiated filipin to one side of the films. However, amphotericin B and filipin and its components, when added to one side of lecithin–cholesterol bilayer films, are able to reduce their resistances. The order of activity of the filipins in lowering the membrane resistances (at a concn. of 10^{-7} M) is filipin II > filipin III > filipin complex, which parallels the order of their lytic action towards lecithin–cholesterol bilayers. On the other hand, in hemolysis tests[12] filipin II and filipin III are almost equally active, while experiments of DEMEL[14] indicate the filipin III is more active in increasing the surface pressure of lecithin–cholesterol monolayers than filipin II. Thus some minor discrepancies remain in the responses of natural and artificial membranes to the filipin components.

It should be mentioned that the effects measured with etruscomycin, pimaricin, both the filipin derivatives (perhydrofilipin and irradiated filipin), and filipin complex and its components do not depend upon the manner of adding the antibiotic, but the effects measured with nystatin and amphotericin B do. Thus, addition of nystatin to one side of the lecithin–cholesterol bilayer membranes does not affect the electrical resistance, while addition to both sides reduces it by a factor of 10^3. Furthermore, addition of amphotericin B to one side of these bilayer membranes reduces their resistance by a factor of 10, while addition of this antibiotic to both sides of the films shows variable effectiveness in lowering the resistance. This last observation is not in agreement with the findings of ANDREOLI AND MONAHAN[17] and those of FINKELSTEIN AND CASS[18], but the results presented for nystatin, when it was added to both sides of the bilayer membranes confirmed their findings. Although it is hard to understand why nystatin only reduces the lecithin–cholesterol bilayer resistance when added to both sides of the film and amphotericin B only shows clear effects when added to one side, one may speculate that the size and form of the antibiotic micelles plays a very important part in the effect of antibiotics on the resistance of lipid bilayers.

In all experiments discussed above, diffusion of bulky antibiotic micelles towards the bilayer films must play an important role, since the antibiotics are added to the aqueous phase bathing the membranes. To avoid the slow diffusion process, attempts were made to dissolve the polyenes in the film-forming solution. In this type of experi-

ment, where the antibiotics were present in the black film, filipin III appeared to be more active in lowering the d.c. resistance of lecithin–cholesterol bilayers than filipin II. This result concurs with the observations of DEMEL[14] but is in contrast to the data obtained when these antibiotics were added outside the films.

It can be concluded that the disruption of bimolecular lipid films and the reduction of the d.c. resistances of membranes by polyene antibiotics correspond with their physiological activities, provided that they are added at low concentrations. At these concentrations, the sensitivity of the membranes towards the antibiotics is determined by the presence of cholesterol. In the experiments discussed so far, 0.1 M NaCl was the aqueous phase bathing the bilayer membranes.

The findings of BUTLER *et al.*[19] and those of NORMAN[20,41] made it of interest to investigate whether or not a certain ion specificity could be determined for filipin complex and other polyene antibiotics acting on bimolecular lipid membranes, and whether or not the presence of cholesterol would be a prerequisite for this effect. The experiments carried out with filipin complex, filipin II, and filipin III which are uncharged polyenes[11], show that the d.c. resistance of lecithin–cholesterol bilayers is appreciably reduced in the presence of Ca^{2+}, but not in the presence of a variety of other cations and anions. The transference number measurements demonstrate that these antibiotics produce cation-selective lecithin–cholesterol bilayers which are far more permeable to Ca^{2+} than to the other cations tested. Thus, it may be concluded that the increase in the conductance of lecithin–cholesterol bilayers in the presence of either filipin complex, filipin II, or filipin III is specific for Ca^{2+}. Filipin complex and filipin III show far less pronounced effects than filipin II.

It should be mentioned that our results for filipin complex differ from those of ANDREOLI AND MONAHAN[17] and those of FINKELSTEIN AND CASS[18]. No ion selectivity has been observed by these authors; only a destruction of the lecithin–cholesterol bilayers after the introduction of the antibiotic was noted.

Nystatin and pimaricin, which are known to be charged, amphoteric polyenes[34,35], show the same specificity for Ca^{2+} in lowering the d.c. resistance of lecithin–cholesterol bilayer membranes. Addition of nystatin to one side of these bilayers renders them cation selective, as does addition to both sides when the membranes are formed in either $CaCl_2$, $MgCl_2$, or $La(NO_3)_3$. However, addition of nystatin to both sides of membranes formed in either NaCl or $NaNO_3$, renders them anion selective. This last observation is in good agreement with the earlier findings of other authors[17,18].

From the results of transference number measurements carried out on lecithin–cholesterol membranes containing either nystatin or filipin complex, it is clear that filipin complex produces cation-selective membranes. This is consistent with the data obtained when the antibiotic is added to the aqueous phase bathing the films. Nystatin produces anion-selective membranes, but the degree of anion selectivity is larger in NaCl than in $CaCl_2$, *i.e.* the membranes are more permeable to Ca^{2+} than to Na^+.

Amphotericin B, which can be dissolved in water only after conversion to the HCl salt, shows a reduction of the d.c. resistance of lecithin–cholesterol bilayer membranes in the presence of several anions, but not in the presence of Ca^{2+} or other cations. This is in agreement with the proposals that amphotericin B-treated membranes are anion selective (*cf.* refs. 17, 18).

The d.c. resistances of bilayers prepared from lecithin alone are not reduced

when the bilayers are treated with filipin complex, its components, nystatin, or pimaricin in the presence of Ca^{2+}, nor after treatment with amphotericin B in the presence of anions. Thus, cholesterol appears to be a prerequisite for the ionic effects measured.

Figs. 5A and 5B compare the effects of the various polyene antibiotics and Ca^{2+} on the d.c. resistance of lecithin–cholesterol bilayer membranes. From Fig. 5B it is obvious that the filipin components I and IV hardly affect the d.c. resistance of lecithin–cholesterol bilayers, even in the presence of Ca^{2+}. This result confirms the findings of SESSA AND WEISSMANN[12].

Addition of filipin complex to one side of lecithin–cholesterol bilayers, followed by addition of Ca^{2+} to the other side, results in a reduction in their resistances only after more than 60 min, while addition of this antibiotic *plus* Ca^{2+} to the same side of the membranes results in a decrease in their resistances within 15 min. We believe that in the first case the permeability of the antibiotic is the limiting factor in the resistance-lowering process.

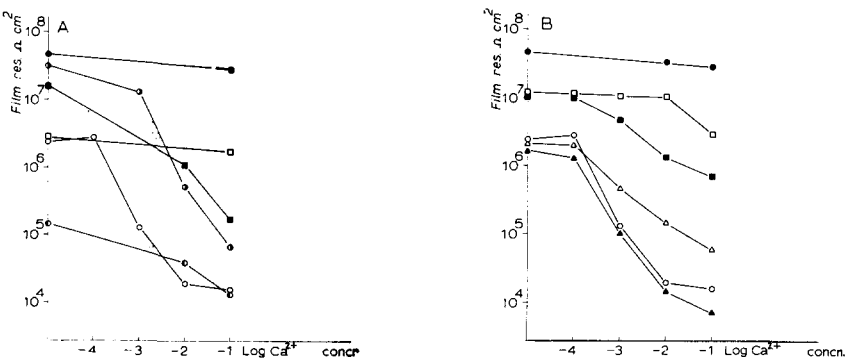

Fig. 5. A. The effect of $4.0 \cdot 10^{-7}$ M polyene antibiotic on the d.c. resistance of lipid bilayer membranes in the presence of Ca^{2+}. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions. Filipin complex (○), pimaricin (■), and amphotericin B (□) were added to one side of the film, while nystatin was added either to one side (◑) or to both sides (◐). The d.c. resistance of the films when $CaCl_2$ but no antibiotic was added is denoted by ●. B. The effect of $4.0 \cdot 10^{-7}$ M filipin complex (○), filipin I (■), filipin III (△), or filipin IV (□) and $4.0 \cdot 10^{-8}$ M filipin II (▲) on the d.c. resistance of lipid bilayer membranes in the presence of Ca^{2+}. The membranes were generated from (1,2-dioleoyl)-3-lecithin/cholesterol 1:1 molar solutions. The d.c. resistance of the films when $CaCl_2$ but no antibiotic was added is denoted by ●.

Our findings concerning the effects of polyene antibiotics on Ca^{2+} transport across lipid bilayer membranes concur with those of NORMAN[20,41] on the effects of antibiotics on the Ca^{2+} flux of ileal tissue. However, this author has presented evidence which suggests that filipin (complex) is able to alter the structure of the microvillous membranes of vitamin D-deficient chick intestines, thus activating a primarily inactive Ca^{2+} transport system. Although the lecithin–cholesterol bilayer membranes are devoid of such a transport system, they apparently show a high permeability to Ca^{2+} when treated with polyenes.

The transference number calculations are based on the assumptions that electric charge is transported by ions and that the driving force for this transport is a difference in salt concentration or electrical potential in the two aqueous phases surrounding the membranes. The mechanism by which ions are transported across lipid bilayer

membranes has not been elucidated. The decrease in the d.c. resistance of lecithin–cholesterol bilayers in the presence of polyene antibiotics and Ca^{2+} can possibly be explained by the formation of fixed sites or pores within the membrane phase or by the functioning of the polyenes as carriers. The first possibility is supported by the electron microscopic studies of KINSKY *et al.*[16,36]. These authors indicate, using negative-staining techniques, that lytic concentrations of filipin complex may produce circular pits approx. 125 Å in diameter, in either mammalian erythrocyte membranes or cholesterol–lecithin dispersions. Further support for this idea is given by the bilayer experiments of ANDREOLI and co-workers[17,37], and those of FINKELSTEIN AND CASS[18]. ANDREOLI *et al.*[37] have pointed out that the interaction of amphotericin B with membrane-bound cholesterol results in the formation of pores whose equivalent radii are in the range of 7–10.5 Å. From the fact that nystatin-treated bilayer membranes are impermeable to glucose but slightly permeable to erythritol, FINKELSTEIN AND CASS[18,22] have estimated that this antibiotic forms aqueous pores with radii of approx. 4 Å. However, HLADKY AND HAYDON[38] have found that nystatin does not give rise to fluctuations in the conductance of lecithin–cholesterol bilayer membranes. These authors conclude from their data that if nystatin forms aqueous pores, they remain open for less than 100 msec.

It is also possible that the polyene antibiotics act as ion carriers as demonstrated for valinomycin, stimulating the transport of K^+ in liposomes[39], bilayer membranes[40] and in the bulk phase[30,31]. The results compiled in Table IV show that some polyene antibiotics (filipin complex and nystatin) are lipid soluble. This fulfills the concept of LIBERMAN[40] concerning ion carriers.

At present, the possibility that some polyene antibiotics act on biological and artificial membranes by forming aqueous pores while other function as ion carriers cannot be excluded. It is hoped that experiments which are presently in progress will permit a distinction to be made between these two alternatives.

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