The Interaction of Polyene Antibiotics with Thin Lipid Membranes

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ABSTRACT Optically black, thin lipid membranes prepared from sheep erythrocyte lipids have a high dc resistance ($R_m \cong 10^8$ ohm-cm^2) when the bathing solutions contain NaCl or KCl. The ionic transference numbers (T_i) indicate that these membranes are cation-selective ($T_{Na} \cong 0.85$; $T_{Cl} \cong 0.15$). These electrical properties are independent of the cholesterol content of the lipid solutions from which the membranes are formed. Nystatin, and probably amphotericin B, are cyclic polyene antibiotics containing $\approx$36 ring atoms and a free amino and carboxyl group. When the lipid solutions used to form membranes contained equimolar amounts of cholesterol and phospholipid, these antibiotics reduced R_m to $\approx 10^2$ ohm-cm^2; concomitantly, T_{Cl} became $\approx$0.92. The slope of the line relating log R_m and log antibiotic concentration was $\approx$4.5. Neither nystatin (2×10^{-5} M) nor amphotericin B (2×10^{-7} M) had any effect on membrane stability. The antibiotics had no effect on R_m or membrane permselectivity when the lipids used to form membranes were cholesterol-depleted. Filipin (10^{-5} M), an uncharged polyene with 28 ring atoms, produced striking membrane instability, but did not affect R_m or membrane ionic selectivity. These data suggest that amphotericin B or nystatin may interact with membrane-bound sterols to produce multimolecular complexes which greatly enhance the permeability of such membranes for anions (Cl^-, acetate), and, to a lesser degree, cations (Na^+, K^+, Li^+).

INTRODUCTION

The polyene antibiotics comprise a group of structurally related, amphipathic cyclic compounds which are produced by a number of *Streptomyces* species (1), and have rather similar biological effects. The characterized polyene antibiotics are cyclic lactones having a molecular weight of 500–1300, and containing a number (usually 4–7) of conjugated —C=C— double bonds in the ring structure (1, 2). Ordinarily, there are approximately twice as

many hydroxyl group substituents as there are double bonds in the ring (2). In general, these drugs may be classified into two groups, depending on the number of carbon atoms in the molecule and the number of ring atoms in the structure (3, 4). Some of the polyene antibiotics (e.g., nystatin or amphotericin B) have carboxyl and amino group substituents, and hence are potential zwitterions (2), while others (e.g., filipin) have no charged substituents and are relatively nonpolar (5).

With few exceptions (6), the effects of these antibiotics on biological systems are referable to the interaction of these compounds with membrane-bound sterols (4, 7). Yeasts and fungi contain substantial amounts of sterols in their cellular membranes, while bacteria do not (4). Thus, amphotericin B is clinically effective in the treatment of systemic mycotic infections (8), and nystatin is clinically useful as a topical antifungal agent (9), while both drugs have no in vivo antibacterial effects (4, 7). In vitro, the polyene antibiotics are bound to, and inhibit the growth of those organisms the cell membranes of which contain sterols, such as fungi (10–13), yeast (14–18), or protozoa (19), but are inactive against, and not bound to bacteria (13, 14, 20). Similarly, the polyene antibiotics inhibit the growth of *Mycoplasma* organisms only when the latter are cultured in the presence of cholesterol (21, 22). In addition, the drugs can increase cation permeability (23) or produce hemolysis (24, 25) in mammalian erythrocytes, and are able to penetrate cholesterol-containing monolayers (26). Since the polyene antibiotics do not affect cell-free systems (27–30), it has been suggested (4, 7) that the biologic effects of these compounds are referable to the increased cellular permeability to a variety of solutes (11, 12, 31, 32) which results from the interaction of these drugs with membrane-bound sterols. However, the nature of such interactions and the mechanisms producing these permeability changes are not clear.

Our interest in these compounds was stimulated by the report of van Zutphen et al. (33), who demonstrated that certain polyene antibiotics produced a marked instability of thin lipid membranes separating two aqueous phases, when the membranes were formed from cholesterol-containing lipids. Thus, it seemed possible that concentrations of the drugs might be chosen which were inadequate to rupture similar membranes, but which would alter their ionic permeability properties. This paper reports the results of such experiments. The data indicate that certain high molecular weight (ca. 1,000) polyene antibiotics with polar substituents (e.g., nystatin and amphotericin B) radically alter the ionic permeability properties of thin lipid membranes separating two aqueous phases. On the other hand, filipin, a lower molecular weight (571), neutral polyene, disrupts the architecture of these membranes without altering their ionic permeability properties. The data are in accord with the results observed when more complex systems,

such as fungi (10, 11), erythrocytes (23–25), yeast (31, 32), or the toad bladder (34), are exposed to these agents. To the extent that these studies with thin lipid membranes are applicable to biological systems, they provide a relatively simple system for evaluating the interactions between polyene antibiotics and membrane-bound sterols. A preliminary report of these studies has appeared elsewhere (35).

METHODS

Optically black, thin lipid membranes separating two aqueous phases were formed from high-potassium (*H*K) sheep red blood cell lipids dissolved in decane (36, 37).

TABLE I
COMPOSITION OF THE LIPID EXTRACT

Preparation	Phospholipid	Cholesterol	Cholesterol (M)/ Phospholipid (M)
	%	%	
XII-7	61.5	25	0.81
XII-7Ac	92	0.1	0.01

The percentage composition of cholesterol and phospholipid is listed for a typical high-potassium (*H*K) sheep red blood cell lipid extract (XII-7) and for the same extract after treatment with acetone (XII-7Ac). The amount of cholesterol and lipid phosphorus in the extracts was estimated as previously described (37), and the phospholipid content of the extracts was computed by assuming the molecular weight of phospholipid to be 750 (37, 39). Experimental details are in Methods.

The experimental methods, their justification, and the properties of the membrane system have been presented in detail in previous publications (37, 38). Except for the modifications described below, these techniques, analytical determinations, and reagents were employed without change in the present studies.

Lipids were extracted from *H*K sheep red blood cells as described previously (37). The extracts, containing cholesterol and phospholipid in a molar ratio of approximately 0.8–1.2 ([37, 39]; Table I) were dissolved in redistilled chloroform at a concentration of approximately 15–40 mg total lipid per ml of chloroform. The lipid extracts were then depleted of cholesterol by acetone extraction in the following manner.[1] 20 volumes of cold (0–4°C) acetone were added to a given volume (1–10 ml) of the lipids dissolved in chloroform. A flocculent precipitate was formed instantaneously, and the resulting suspension was incubated at −10°C for approximately 20 min. The suspension was then centrifuged for approximately 10 min at 5000 *g* at 4°C. The supernatant fluid was discarded; the pellets were dissolved in redistilled chloroform at approximately 30 mg lipid per ml of chloroform, and stored at − 15°C under N_2. Table I illustrates the composition of a typical lipid extract of *H*K sheep red blood cell lipids before and after acetone extraction. In agreement with previous

[1] We are indebted to Dr. Athos Ottolenghi for suggesting this modified procedure for us.

studies (37), the acetone-treated lipids contained negligible amounts of cholesterol. Table I also shows that phospholipids comprised the major fraction, by weight, of the acetone-treated lipids.

The solutions used to form thin lipid membranes (membrane solutions) contained approximately 25–45 mg of acetone-extracted *HK* sheep red blood cell lipid per ml of decane. Varying amounts of cholesterol (Calbiochem, Los Angeles, Calif.), indicated in the text, were added to the membrane solutions. The conditions for generating thin lipid membranes separating two aqueous phases were identical with previous studies (37, 38). The membrane solutions were applied with a brush technique (40) to an aperture (1–3 mm) on a polyethylene diaphragm separating two chambers (front and rear), each of which contained an aqueous phase. All experiments were carried out at room temperature (22–24°C), and the aqueous solutions were unbuffered. The pH of the aqueous solutions was approximately 5.8, except when the aqueous phases contained Na acetate, when it was approximately 6.8. Under these conditions, the membranes formed were optically black and stable for approximately 1–3 hr (37). Membrane thicknesses, estimated by electrical methods (41), were in the range 50–150 A (36).

The electrical properties of the membranes, including dc resistances (R_m), zero-frequency capacitances (C_m), and membrane voltages (V_m) were recorded as previously described (36, 37). The dc circuit was arranged so that the rear chamber was positive or negative, and the front chamber was grounded.

An estimate of the relative ionic permeabilities of the membranes was made by computing the ionic transference number (T_i) of a given ionic species (defined as G_i/G_m, where G_i is the membrane conductance of the *i*th ion, and G_m the total membrane conductance) from the steady-state membrane potential (V_m) when the two aqueous phases bathing the membrane contained unequal concentrations of a single salt (37). The utilization of this approach depends on certain assumptions, namely, that electric charge traverses a given membrane only in ionic form and that the only significant driving forces for ion transport are differences in salt concentration or electrical potential in the two aqueous phases bathing the membrane (37). The validity of these assumptions, although established under the given experimental conditions for the untreated (37) or valinomycin-exposed membrane systems (38), is limited to restricted conditions when the membranes have been exposed to solutions containing polyene antibiotics (cf. Fig. 7 and Discussion, below).

Nystatin (Batch No. 46290-039) and amphotericin B (Type 1; Batch No. 38675-001) were kindly furnished by Miss Barbara Stearns, Squibb Institute for Medical Research, New Brunswick, N. J. Filipin (96% pure; Reference No. 8393-DE6-11-8) was kindly provided by Dr. G. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich. The molecular weights of the antibiotics, supplied by the manufacturers, were as follows: nystatin, 932; amphotericin B, 959; filipin, 571. The antibiotics were stored as the dry powder in the dark at 4°C. On the day of an experiment, a stock solution ($\leq$0.5 mg/ml) of the antibiotic dissolved in methanol was prepared, and appropriate aliquots of the stock methanolic solution were added to the aqueous solutions less than an hour prior to use. The stock solutions were discarded within 8 hr after preparation to minimize experimental variation due to inactivation of the antibiotics in solution.

RESULTS

Effect of Nystatin on Membrane Stability and dc Resistance

In the absence of nystatin, the dc resistances (R_m) of planar thin lipid membranes prepared from sheep red blood cell lipids are in the range 1–3 × 10^8 ohm-cm^2, when the aqueous phases bathing these membranes contain 0.1 M NaCl or KCl, and are stable for the duration of the membranes ([37]; Fig. 1). A typical experiment illustrating the effect of nystatin (aqueous phase) on the dc membrane resistance is shown in Fig. 1. The molar ratio of cho-

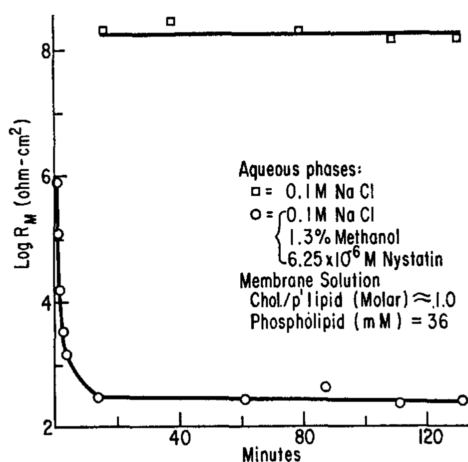


FIGURE 1. The effect of nystatin on the dc resistance (R_m) of thin lipid membranes. The molar ratio of cholesterol to phospholipid (chol./p'lipid) in the membrane solution was approximately 1. The aqueous solutions (pH ≈ 5.6) bathing the membrane contained 0.1 M NaCl (□) or 0.1 M NaCl, 1.3% methanol, and 6.25 × 10^{-6} M nystatin (○). For experimental details, see Methods and the text.

lesterol to phospholipid in the membrane solution was approximately 1. When nystatin (6.25 × 10^{-6} M) was present in the aqueous phases, there was a prompt reduction in the dc membrane resistance; after 20 min, the resistance had fallen to approximately 4 × 10^2 ohm-cm^2, and was stable for the duration of the membrane. However, the antibiotic had no detectable effect on membrane duration.

It should be noted that the criterion for membrane stability is arbitrary. Under the present experimental conditions, the formed membranes were usually stable for 45 min to 5 hr, with an average life-span of 1–2 hr (37). Rupture of the membranes occurred because of technical difficulties, experimental error, or undue vibration of the experimental apparatus, but was often due to undetermined causes. We have adopted a duration of 1 hr or

more, for an optically black membrane, as a reasonable criterion (33), under our experimental conditions, for membrane stability. According to this convention, nystatin (aqueous phase) had no detectable effect, in the concentration range 10^{-8}–2×10^{-5} M, on the stability of at least 80 membranes. van Zutphen et al. (33) observed that nystatin (approximately 4×10^{-5} M) shortened the life-span of cholesterol-lecithin membranes approximately 50%.

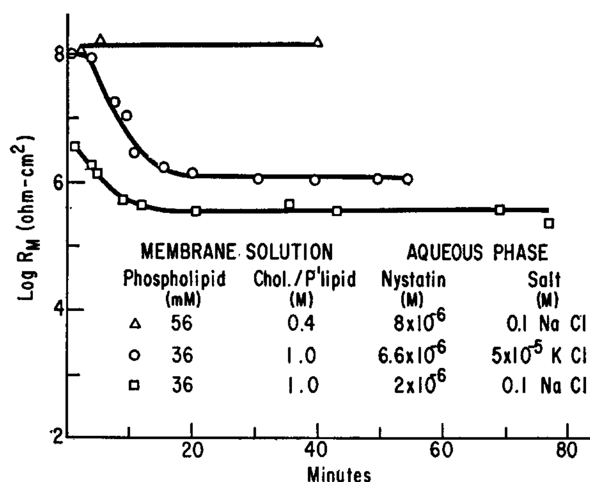


FIGURE 2. The time course of the dc membrane resistances (R_m) under different suboptimal conditions for the nystatin-dependent reduction in R_m. With reference to Fig. 1 (lower curve), either the molar ratio of cholesterol to phospholipid (chol./p'lipid) in the membrane solution (△), or the salt concentration (○), or nystatin concentration (□) in the aqueous phases bathing the membranes was reduced (as indicated in the figure). Experimental details are in Methods or the text.

The nystatin-dependent reduction in dc membrane resistance (Fig. 1) involved potential interactions among a number of variables, including the composition of the membrane solution, and the salt, methanol, and nystatin concentrations in the aqueous phases bathing the membrane. An evaluation of the system required that the dc membrane resistances were independent of time, when the concentration of a single variable in the nystatin-dependent reduction in membrane resistance was less than optimal, with reference to Fig. 1. Three such experiments are illustrated in Fig. 2. The dc membrane resistance was unaffected by nystatin (aqueous phase, 8×10^{-6} M) when the molar ratio of cholesterol to phospholipid in the membrane solution was 0.4. Similarly, when the nystatin concentration was reduced (aqueous phase, 2×10^{-6} M) or the salt concentration in the aqueous phases was altered (5×10^{-5} M KCl), the reduction in membrane resistance was considerably less than that observed in Fig. 1. Furthermore, in each instance, reasonably

constant values for membrane resistances were obtained, which persisted for the duration of the membrane. In the studies to be described below, stable membrane resistances, such as those illustrated in Figs. 1 and 2, were observed under the various experimental conditions.

The effect of different nystatin concentrations (aqueous phases) on the dc membrane resistance, when the composition of the membrane solutions

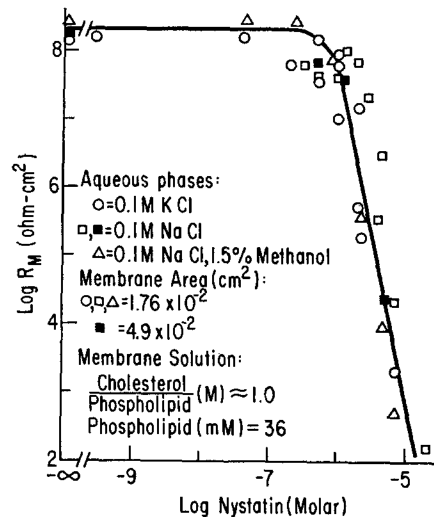


FIGURE 3. The relation of the log membrane resistance (R_m, ordinate) to the log nystatin concentration (M) in the aqueous phase (abscissa). The molar ratio of cholesterol to phospholipid in the membrane solutions was approximately 1 in all instances. The aqueous phases contained 0.1 M KCl (○), 0.1 M NaCl, (□, ■), or 0.1 M NaCl, 1.5% methanol (△). The area of the planar membranes, indicated in the figure, was varied by using polyethylene diaphragms with apertures of different diameter. Experimental details are in Methods.

was held constant (the molar ratio of cholesterol to phospholipid was approximately 1) is illustrated in Fig. 3. The membrane resistance was independent of the nystatin concentration until the latter exceeded 10^{-7} M. In the concentration range 10^{-6}–10^{-5} M nystatin, the relationship between the logarithm of membrane resistance and the logarithm of nystatin concentration had a slope of approximately minus 4.5. Within the limits of experimental error, the results were independent of the cation (K^+ or Na^+) in the aqueous phase. Furthermore, the effect was not due to the methanol in the aqueous solutions since the same concentrations of methanol, when nystatin was absent or present in low concentrations (Fig. 3, triangles), did not affect membrane resistance.

The possibility that the nystatin-dependent reduction in the dc membrane resistance (Figs. 1, 3) was due to electrical "leakage" pathways be-

tween the edges of the membranes and the supporting polyethylene framework could not be absolutely excluded. However, as illustrated in Fig. 3, the dc membrane resistance was directly proportional to membrane area over nearly a threefold change in the latter. In contrast, if the main pathway conducting electrical charge were through border conductances at the edges of the membranes, the dc resistance should vary with the circumference, rather than the area, of the membranes (42).

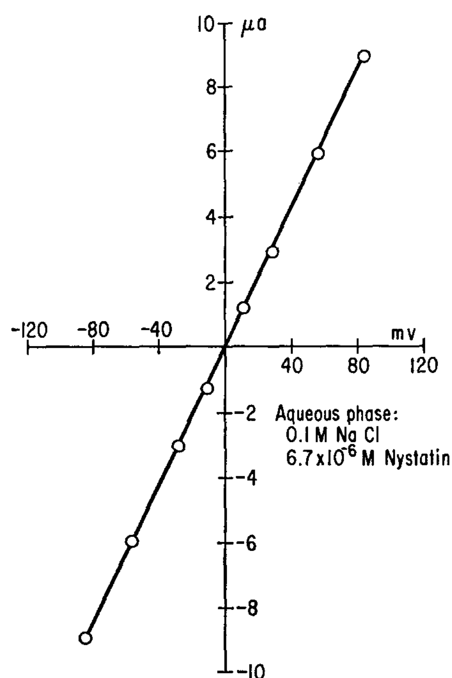


FIGURE 4. The relationship of current (ordinate) to voltage (abscissa) for nystatin-exposed, low dc resistance thin lipid membranes. The molar ratio of cholesterol to phospholipid in the membrane solutions was approximately 1, and the aqueous phases contained 0.1 M NaCl, 6.7×10^{-6} M nystatin. Experimental details are in Methods.

As shown in Fig. 4, the current-voltage properties of the nystatin-treated, low dc resistance membranes were ohmic over a minimum range of ± 85 mv when the aqueous phases bathing the membrane were identical in composition.

Fig. 5 illustrates the relationship of the nystatin-dependent reduction in membrane resistance to the concentration of salt in the aqueous phases bathing the membranes when the molar ratio of cholesterol to phospholipid in the membrane solution was 1. In the absence of nystatin (Fig. 5, closed symbols), the dc membrane resistance was independent of the salt concentration (in the range 10^{-4}–10^{-1} M) or the cation (Na^+ or K^+) in the aqueous phase When nystatin (6.7×10^{-6} M) was present in the aqueous phases bathing the membranes (Fig. 5, open symbols), the relationship between the logarithm of membrane resistance and the logarithm of the salt

concentration (in the range 10^{-4}–10^{-1} M) was linear, with a slope of approximately minus one, and was also independent of the cation (Na^+ or K^+) in the aqueous phase.

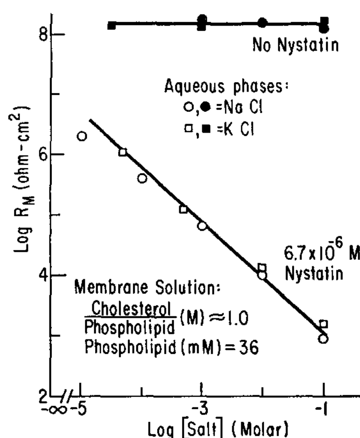


FIGURE 5. The effect of varying salt concentrations in the aqueous phases bathing the membranes on the dc resistance of thin lipid membranes in the presence (open symbols) and absence (closed symbols) of nystatin (aqueous phase = 6.7×10^{-6} M). The molar ratio of cholesterol to phospholipid in the membrane solutions was approximately 1. Experimental details are in Methods.

The Effect of Lipid Composition

At present, there is no direct method available for estimating the cholesterol content of thin lipid membranes. However, it seems probable that the cholesterol content of the membrane solutions determines, at least to a limited degree, the cholesterol content in the membranes. Thus, the zero-frequency capacitance of cholesterol-lecithin membranes increases when the molar ratio of cholesterol to phospholipid in the membrane solution exceeds 1.0 (43). Similarly, the addition of cholesterol to the membrane solutions alters both the water permeability (44) and the permeability to organic solutes (45) of thin lipid membranes separating two aqueous phases. In addition, as indicated earlier, van Zutphen et al. (33) noted polyene-dependent membrane instability only when the membranes were formed from cholesterol-containing membrane solutions.

However, in the absence of polyene antibiotics in the aqueous phases, both the total membrane conductance and the ionic selectivity properties of thin lipid membranes prepared from sheep red cell lipids are independent of the molar ratio of cholesterol to phospholipid (in the range 0–1.2) in the membrane solutions (37). Similarly, the striking degree of potassium, with respect to sodium, selectivity produced by valinomycin in these (38) and

other (46, 47) similar membranes is independent of the cholesterol content of the membrane-forming solutions. In contrast, Fig. 2 shows that the nystatin-dependent increase in membrane conductance (e.g., Fig. 1) was related to the sterol content of the membrane solutions.

This phenomenon is illustrated more completely in Fig. 6. In these experiments, the composition of the aqueous phases was kept constant (0.1 M NaCl, 1.5% methanol, 6.8×10^{-6} M nystatin). When the membrane solutions contained equimolar amounts of sterol and phospholipid, these conditions were sufficient to produce at least a hundred thousandfold reduction in dc membrane resistance (Fig. 3). However, as shown in Fig. 6, nystatin had no effect on the dc membrane resistance when the molar ratio of cholesterol to phospholipid in the membrane solutions was less than approximately 0.5. When this ratio was exceeded, the fall in the electrical resistance of the membranes was proportional to the molar ratio of cholesterol to phospholipid (in the range 0.5–1.0) in the membrane solutions. Furthermore, additional increments in this molar ratio (i.e., greater than 1) had negligible

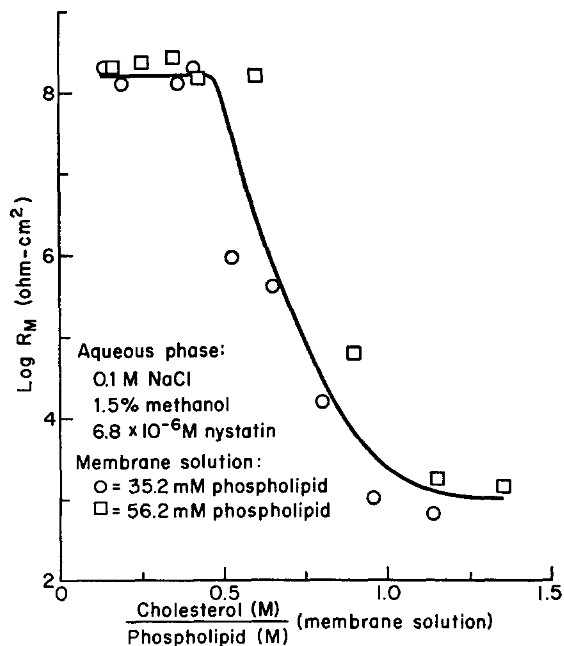


FIGURE 6. The relation of the nystatin-dependent reduction in dc membrane resistance (R_m) to the molar ratio of cholesterol to phospholipid in the membrane solution. The aqueous phases contained 0.1 M NaCl, 1.5% methanol, and 6.8×10^{-6} M nystatin. Thin lipid membranes were formed from membrane solutions containing 35.2 mM (○) or 56.2 mM (□) phospholipid and the indicated amount of cholesterol. Experimental details are in Methods.

effects on membrane resistance. The results were approximately the same (within the precision of the experimental methods) over nearly a twofold range in the phospholipid concentration (35.2–56.2 mM) in the membrane

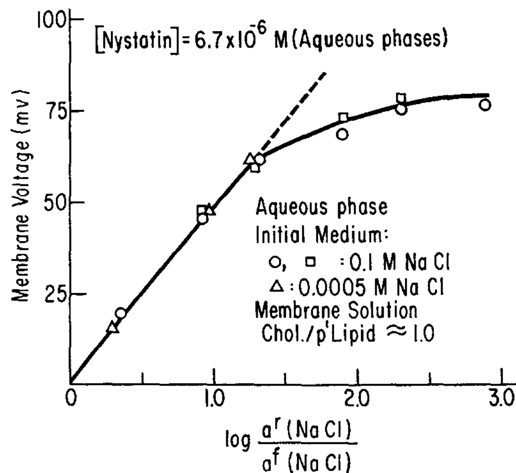


FIGURE 7. The relationship of membrane potential (V_m) to the logarithm of the activity ratio of NaCl [log a^r(NaCl)/a^f(NaCl)] in the two aqueous phases bathing nystatin-treated, low dc resistance membranes. The aqueous phases all contained the indicated concentration of nystatin, and the molar ratio of cholesterol to phospholipid (chol./p'lipid) in the membrane solutions was approximately 1. When the membranes were formed in 0.1 M NaCl, (○ or □), solutions of increasing dilution were introduced into the front chamber; in certain experiments (□), the osmolality of the solutions bathing the membrane was kept constant by addition of sucrose to the dilute NaCl solutions. When the membranes were formed in 0.0005 M NaCl (△), solutions of increasing concentration were introduced into the front chamber. In the latter case, V_m was negative, and both sides of Equation 1 were multiplied by minus one and the data was plotted as shown in the figure. Each point represents the mean of at least three separate measurements of V_m on at least two different membranes. Experimental details are in Methods. Osmotic coefficients were obtained from Robinson and Stokes (48) and activity coefficients from Latimer (49).

solutions. Consequently, the effect seemed related, for the most part, to the fractional, rather than the absolute, cholesterol content of the membrane solutions.

Effect of Nystatin on Membrane Permselectivity

In the absence of polyene antibiotics, thin lipid membranes prepared from sheep red blood cell lipids are predominantly cation-selective (37). Their permselectivity has been rationalized in terms of the negatively charged polar groups of the phospholipids present in sheep red cell lipids (39), which presumably are oriented on the surface of these membranes. However, when

TABLE II

THE EFFECT OF NYSTATIN ON THE PERMSELECTIVITY OF THIN LIPID MEMBRANES

Membranes	Membrane solution		Aqueous phase						
(number)	P'lipid	Chol. (M)/p'lipid (M)*	Rear chamber	Front chamber	Nystatin	R_m	V_m	T_+	T_{Cl}
	mM			M		ohm-cm²	mv		
7	24–56	≤0.4	0.1 NaCl	0.01 NaCl	0	2.8±1.1×10⁸	−36±4.0 (10)	0.83	0.17
6	24–56	≤0.4	0.1 NaCl	0.01 NaCl	8×10⁻⁶	2.1±0.9×10⁸	−40±7.0 (8)	0.87	0.13
19	24–56	≈1.0	0.1 NaCl	0.01 NaCl	0	2.2±0.9×10⁸	−34±6.0 (34)	0.81	0.19
19	24–56	≈1.0	0.1 KCl	0.01 KCl	0	2.0±0.8×10⁸	−37±4.0 (38)	0.84	0.16
15	24–56	0.8–1.4	0.1 NaCl	0.01 NaCl	6–8×10⁻⁶	4.0±3×10³	+46±5.0 (23)	0.08	0.92
2	35	1.0	0.1 KCl	0.01 KCl	6.7×10⁻⁶	5.0×10³	+47 (3)	0.07	0.93

Thin lipid membranes were formed from membrane solutions whose composition is listed in the table. Nystatin, when indicated, was in all the aqueous phases. Membrane resistances (R_m) were measured when the front and rear chambers contained the salt concentration listed for the rear chamber, and are expressed as the mean ± standard deviation (SD) for the indicated number of membranes. The membrane potentials (V_m) were recorded when the aqueous phases bathing the membranes contained the salt concentrations indicated in the table, and are expressed as the mean ± SD. The number in parentheses indicates the number of observations. The transference numbers were computed from V_m (37). Experimental details are in Methods and the text.

* Chol., cholesterol; p'lipid, phospholipid.

the membranes were exposed to aqueous solutions containing nystatin, their permselective properties were strikingly altered *pari passu* with the increases in membrane conductance already presented (Figs. 1–6).

The computation of ionic transference numbers (T_i) from V_m, the steady-state membrane potential, depends on certain assumptions, discussed previously (37) and above (cf. Methods). When NaCl is the only salt in the solutions bathing the membrane, the equation (37) relating membrane potential (V_m) to the activity of NaCl in the aqueous phases becomes:

TABLE III

THE EFFECT OF NYSTATIN ON THE PERMEABILITY OF THIN LIPID MEMBRANES TO LARGER IONS

Salt (aqueous phase)	Cation diameter		Anion diameter		R_m	V_m	T_+	T_-
	Crystallographic	Corrected hydrated	Crystallographic	Corrected hydrated				
	A		A		ohm-cm²	mv		
LiCl	~1.4	6.8	3.62	4.28	4.7×10^2	33	0.2	0.8
$NaCH_3COO$	1.94	5.52	~6.0	7.4	0.8×10^2	49	0.06	0.94

The molar ratio of cholesterol to phospholipid in the membrane solutions was approximately 1. The aqueous phases contained 6.7×10^{-6} M nystatin. When acetate was present, the pH of the aqueous phases was ~6.8. Otherwise, the pH of the aqueous phases was ~5.8. When membrane resistances (R_m) were measured, the concentration of the indicated salt was 0.1 M in both aqueous phases. Membrane potentials (V_m) were measured when there was a 10-fold difference in the salt concentration (0.1–0.01 M) in the aqueous phases bathing the membranes. The recorded values of R_m are the mean for two different membranes, and of V_m the mean of six different measurements on two separate membranes. The crystallographic ion sizes for all ions and the corrected hydrated diameters for Li^+, Na^+, Cl^- were obtained from Stern and Amis (50). The corrected hydrated diameter for acetate was estimated according to a method suggested by Robinson and Stokes (reference 48, p. 125). The transference numbers (T_+ and T_-) were competed from V_m (37). Experimental details are in Methods.

$$V_m = (4.6 T_{Cl} - 2.3) \frac{RT}{F} \log \frac{a^r(\text{NaCl})}{a^f(\text{NaCl})} \quad (1)$$

where R = gas constant, T = °K, F = Faraday's number, and a^r(NaCl) and a^f(NaCl), the activities of NaCl in the rear and front chambers, respectively. Hence, V_m should be a linear function of the logarithm of the activity ratio of salt in the two aqueous phases bathing the membrane. Such results (when the salt concentration in the aqueous phases was in the range 0.001–0.1 M) have been obtained for these membranes, in the high dc resistance, cation-selective state (37) or after their exposure to valinomycin, when the membranes have a low dc resistance and are highly K^+ selective (38).

Fig. 7 illustrates similar studies when cholesterol-containing, low dc resist-

ance membranes were formed in the presence of nystatin. The plot of V_m vs. the logarithm of the NaCl activity ratio in the two aqueous phases bathing the membranes was linear for only relatively low activity ratios (less than 25). The initial slope was the same when the membranes were formed in dilute (△, 0.0005 M NaCl; Fig. 7) or relatively concentrated (○, □, 0.1 M NaCl; Fig. 7) salt solutions. Furthermore, the deviation from linearity persisted when both aqueous phases were isotonic (□, Fig. 7). From the slope of the linear portion of the curve in Fig. 7 (48 mv) and Equation 1, T_{Cl} was computed to be 0.94, while T_{Na} was 0.06.

Table II summarizes a large number of observations on the dc resistance and ionic selectivity characteristics of thin lipid membranes formed from membrane solutions of varying composition, and the effect of nystatin on these properties. When nystatin was absent, the electrical properties of the membranes were independent of the molar ratio of cholesterol to phospholipid in the membrane solutions (37). In contrast, the combination of nystatin (aqueous phase) and a high molar ratio of cholesterol to phospholipid in the membrane solutions (i.e., greater than 0.8) invariably yielded a low resistance, highly Cl^--selective membrane, regardless of whether the cation in the aqueous phase was Na^+ or K^+. However, T_{Cl} did not become unity, but was approximately 12 times as great as T_+ (T_{Na} or T_K). Since the cations contributed only a small fraction to the total ionic current, the experimental arrangement was not adequate for evaluating whether or not nystatin produced modest degrees of Na^+/K^+ discrimination in these membranes.

Furthermore, as illustrated in Table III, the nystatin-dependent increase in membrane conductance and anion permselectivity was still observed when the larger acetate anion (corrected hydrated diameter $\approx$ 7.4A) was substituted for Cl^- in the aqueous phase. A comparison of Tables II and III indicates that the nystatin-treated membranes were approximately three times more permeable to the larger Li^+ (T_{Li} = 0.2; Table II) than to Na^+ of K^+ ($T_{Na} \cong T_K$ = 0.8; Table II).

The Effect of Amphotericin B and Filipin

Fig. 8A illustrates certain salient features of the nystatin molecule (51). The antibiotic is an interrupted heptaene (mol wt $\cong$ 932), and contains 46–47 C atoms, a 36–37 membered ring, and a free carboxyl and amino group, the latter on a mycosamine (52) moiety. Fig. 8B shows the structure arbitrarily assigned by us to the bracketed moiety in Fig. 8A, for an approximation of molecular size. The inner diameter of the arbitrary nystatin molecule was estimated from Corey-Pauling space-filling models, and varied from approximately 6.5 A to a maximum of 7.5 A, depending on the manner in which the model was rotated.

The heptaene antibiotic amphotericin B (mol wt $\cong$ 959) has an empirical

formula ($C_{46}\,H_{73-75}O_{18-20}N$) similar to that of nystatin, and also contains mycosamine and a free carboxyl group (2). In yeast and fungi (4, 7) or mammalian erythrocytes (24, 25), amphotericin B is a more potent lytic agent than nystatin, but its effects can be differentiated from those of filipin (4). Table IV shows that the effects of amphotericin B on these thin lipid membranes were strikingly similar to those of nystatin (Table II), except that amphotericin B seemed more potent. Thus, amphotericin B (1.67×10^{-7} M) produced both a definite reduction in the dc membrane resistance (0.63×10^2 ohm-cm²) and a high degree of Cl^- selectivity ($T_{Cl} = 0.85$), but

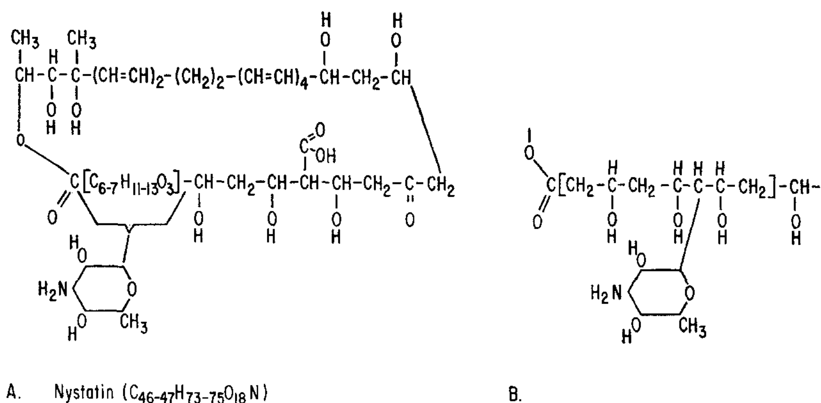


FIGURE 8. A, proposed structure of nystatin (51); B, arbitrarily assigned structure to the bracketed moiety in A for the assembly of Corey-Pauling space-filling models.

only when the membrane solutions contained a substantial fraction of cholesterol. Additionally, the effect of amphotericin B on the electrical properties of the membranes occurred over a relatively narrow concentration range (6×10^{-9}–1.6×10^{-7} M). In this range, there was no detectable effect on membrane stability.

Although both nystatin and amphotericin B greatly increased the Cl^- conductance of cholesterol-containing membranes, they also augmented cation conductance to a considerable degree. As illustrated in Table V, G_{Na}, the membrane Na conductance (computed from the data in Tables II and IV) was increased ten thousand- to a hundred thousandfold in the presence of these antibiotics, despite the fact that T_{Na} was concomitantly reduced (Tables II and IV).

The pentaene filipin is smaller (mol wt, 571; $C_{35}\,H_{58}\,O_{11}$) than either nystatin or amphotericin B, has a 28-membered ring, and has no charged substituents (5). Corey-Pauling space-filling molecular models of the filipin molecule were constructed, and the inner diameter of the ring compound, in a variety of configurations, did not exceed 4.5 A. Filipin is considerably

TABLE IV
THE EFFECT OF AMPHOTERICIN B ON THE PERMSELECTIVITY AND STABILITY OF THIN LIPID MEMBRANES

Membranes (number)	Membrane solution		Aqueous phase			Membrane duration	R_m	V_m	T_{Na}	T_{Cl}
	Phospholipid	Chol. (M)/ P'lipid (M)*	Rear chamber	Front chamber	Amphotericin B					
	mM		M	M	M	*min*	(*ohm-cm²*)	*mv*		
2	35.2	1.02	0.1 NaCl	0.01 NaCl	6×10^{-9}	88 [77–98]	1.7×10^8 [1.32–2.04]	−43 [38–54]	0.89	0.11
2	35.2	1.02	0.1 NaCl	0.01 NaCl	1.6×10^{-7}	97 [77–113]	0.63×10^2 [0.42–0.85]	+38 [35–41]	0.15	0.85
2	40.0	<0.05	0.1 NaCl	0.01 NaCl	1.4×10^{-7}	83 [78–88]	2.4×10^8 [2.2–2.6]	−51 [46–54] (6)	0.96	0.04

Thin lipid membranes were formed from the indicated membrane solutions. Membrane resistances (R_m) were measured when both aqueous phases contained the salt concentration listed for the rear chamber, and membrane potentials (V_m) when the aqueous phases were as indicated. R_m, V_m, and membrane duration are recorded as the mean of a number of observations listed in parentheses. The range of the observations is in brackets. Transference numbers (T_{Na} and T_{Cl}) were computed from V_m (37). Experimental details are in Methods.
* Chol., cholesterol; p'lipid, phospholipid.

more potent than either of the larger polyene antibiotics on biological systems (4, 7). Additionally, there is evidence that filipin can interact with oriented lipid structures that have a low (53) or absent (6) fractional sterol content. Its effect on these thin lipid membranes is summarized in Table VI. In agreement with the observations of others (33), the smaller antibiotic produced a striking instability of membranes formed from lipid solutions having a high (approximately 1) molar ratio of cholesterol to phospholipid. The disruptive effect was evident over a small concentration range of filipin ($>1.75 \times 10^{-6}$–5.2×10^{-6} M). At 5.2×10^{-6} M filipin, membranes could be formed, but the latter produced interference colors only, and became visibly solidified and torn in less than 20 min. At 1.75×10^{-5} M filipin, the

TABLE V

THE EFFECT OF POLYENE ANTIBIOTICS ON THE IONIC CONDUCTANCES OF THIN LIPID MEMBRANES

Antibiotic	G_m	G_{Na}	G_{Cl}
		mho-cm^{-2}	
None	4.55×10^{-9}	3.68×10^{-9}	0.87×10^{-9}
Nystatin (6–8×10^{-6} M)	2.5×10^{-4}	2.0×10^{-5}	2.3×10^{-4}
Amphotericin B (1.4×10^{-7} M)	1.6×10^{-2}	2.4×10^{-3}	1.36×10^{-2}

The total ionic conductance (G_m) and the individual ionic conductances (G_{Na} and G_{Cl}) were computed for the indicated conditions from the data in Tables II and IV, and the equations (37) relating ionic transference numbers and membrane conductance.

membranes which were formed solidified almost instantaneously, and tore abruptly. Hence, electrical measurements were not feasible. For similar reasons, the electrical data at 5.2×10^{-6} M filipin are of questionable validity. However, they are in accord with the electrical data at lower filipin concentrations, and indicate that the antibiotic did not alter the relative ionic conductances of the membranes. In addition, concentrations of filipin which were inadequate to produce membrane instability had no detectable effect on the dc membrane resistance. It should be noted that some form of filipin-membrane interaction probably occurred even when the ratio of cholesterol to phospholipid in the membrane solutions was low (<0.05). As indicated in Table VI, although the membranes formed under these conditions were stable at high filipin concentrations (1.7×10^{-5} M), they remained thick and reflected only interference colors with no visible areas that were optically black.

DISCUSSION

The high electrical resistance of thin lipid membranes separating two aqueous phases, ([37, 41]; Fig. 1; Table II), in their native state, may be referable

TABLE VI

THE EFFECT OF FILIPIN ON THE PERMSELECTIVITY AND STABILITY OF THIN LIPID MEMBRANES

Membranes (number)	Membrane solution: Phospholipid	Membrane solution: Chol.(M)/P'lipid(M)*	Aqueous phase: Rear chamber	Aqueous phase: Front chamber	Aqueous phase: Filipin	Membrane duration	R_m	V_m	T_{Na}	T_{Cl}
	mM			*M*		*min*	*ohm-cm²*	*mv*		
3	35.2	1.02	0.1 NaCl	0.01 NaCl	$\leq 1.75 \times 10^{-6}$	90 [80–104]	1.7×10^{8}	−47 (8)	0.92	0.08
2	35.2	1.02	0.1 NaCl	0.01 NaCl	5.2×10^{-6}	<20 (thick)	4.1×10^{6}	−48 (3)	0.93	0.07
6	35.2	1.02	0.1 NaCl	0.01 NaCl	1.7×10^{-6}	—	—	—	—	—
2	35.2	<0.05	0.1 NaCl	0.01 NaCl	1.7×10^{-6}	62 (thick)	1.6×10^{8}	−44 (3)	0.90	0.10

The conditions and symbols in this table are the same as in Table IV, except that filipin was used in place of amphotericin B. The term "thick" in parentheses in the column listing membrane duration refers to membranes which reflected interference colors and no optically black areas during their duration.

* Chol., cholesterol; p'lipid, phospholipid.

primarily to the properties of the surface monolayers bounding the membranes at the interfaces with aqueous solutions. Presumably, the monolayer configuration imposes relative restrictions on the rotational mobilities of the polar moieties of phospholipids into the dielectric "core" of the membrane[2] (37, 38, 41, 54). Similarly, the cation-selective properties of the high dc resistance membranes (Table II) have been rationalized in terms of fixed negative charges on the membrane surfaces, attributable to the negatively charged phospholipids present in the lipid extracts, which regulate the penetration of ions into the membranes (37). The observations in this paper indicate clearly that, under appropriate experimental conditions, nystatin (Figs. 1 and 3 Tables II, III, and V) and amphotericin B (Tables IV and V) radically reduce the dc resistance, and, concomitantly, alter the ionic selectivity properties of these thin lipid membranes. A reasonable hypothesis is that the interaction of these polyene antibiotics with membrane-bound cholesterol modifies the surface properties of the membranes, and, consequently, their electrical behavior.

Let us assume that m molecules of P, the polyene antibiotic (either nystatin or amphotericin B) may interact with n molecules of C, membrane-bound cholesterol, to form a single unit P_mC_n. Furthermore, p units of P_mC_n may interact with each other to form $(P_mC_n)_p$. Let us also assume that the increase in G_m, the membrane conductance (Table V), is proportional to the concentration of a complex such as $(P_mC_n)_p$ within the membrane, when the aqueous phases bathing the membrane contain 0.1 M salt (NaCl or KCl). Accordingly,

$$G_m = u_1[(P_mC_n)_p] \tag{2}$$

where u_1 is a constant of proportionality.

It is recognized that Equation 2 represents a limiting case, since it is possible that a number of polyene-cholesterol units may exist (i.e., $P_{i \neq m}\ C_{j \neq n}$), each of which may interact to form various species, i.e., $(P_{i \neq m}\ C_{j \neq n})_{r \neq p}$. Consequently, a more general form of Equation 2 is

$$G_m = u_1[(P_mC_n)_p] + u_2[(P_{i_2 \neq m}C_{j_2 \neq n})_{r_2 \neq p}] + u_3[(P_{i_3 \neq m}C_{j_3 \neq n})_{r_3 \neq p}] + \cdots \tag{3}$$

However, in the absence of specific information concerning such units, Equation 2 will be considered.

The interaction between polyene antibiotic and cholesterol may be described as

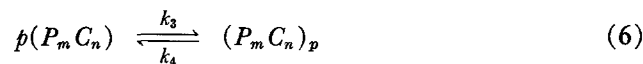
$$mP + nC \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P_m\,C_n\,. \tag{4}$$

[2] Andreoli, T. E., and D. C. Tosteson. Manuscript in preparation.

Furthermore, at equilibrium,

$$[P_mC_n] = K_1[P]^m[C]^n \tag{5}$$

where $K_1 = k_1/k_2$. The formation of a unit such as $(P_mC_n)_p$, responsible for the increase in membrane conductance, may be described as



$$p(P_mC_n) \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (P_mC_n)_p \tag{6}$$

and, at equilibrium,

$$[(P_mC_n)_p] = K_2[P_mC_n]_p \tag{7}$$

where $K_2 = k_3/k_4$. Substitution of Equations 5 and 7 into Equation 2 yields

$$G_m = u_1'([P]^m[C]^n)^p \tag{8}$$

The proportionality constant u_1' in Equation 8 may depend, in part, on the partition coefficient of $(P_mC_n)_p$ between the aqueous and membrane phases, and may also be related to the mobility of $(P_mC_n)_p$ within the membrane (see below).

In Fig. 3, the experimental conditions were such (cf. Figs. 5 and 6) that the reduction in R_m was dependent primarily on the concentration of nystatin in the aqueous phase. Consequently, the slope (minus 4.5) of the relation between the logarithm of membrane resistance and the logarithm of nystatin concentration (Fig. 3) was a measure of $(m \times p)$ in Equation 8. The relatively restricted concentration range over which amphotericin B exerted its effects (Table IV) on the dc membrane resistances implies a similar mode of participation for this antibiotic. According to this view, a single membrane-bound unit which participates in the reduction of membrane electrical resistance might be comprised of a multimolecular aggregate of nystatin (or presumably amphotericin B) with membrane-bound cholesterol. Such a unit might be multimolecular either because a number of polyene antibiotic molecules are involved, either simultaneously or cooperatively, in a single interaction with membrane-bound cholesterol (i.e., m in Equation 8 is greater than one), or because more than one unit of P_mC_n is involved in the formation of a single membrane-bound unit responsible for the increase in membrane conductance (i.e., p in Equation 8 is greater than one), or because of a combination of these two factors.

In a similar manner, the sigmoid curve in Fig. 6 can be rationalized by assuming that $(m \times p)$ in Equation 8 is greater than one. However, it is also possible that cholesterol was not incorporated into the membranes to any significant degree until the molar ratio of cholesterol to phospholipid in the

membrane solution exceeded 0.4–0.5. Alternatively, it may be that the form of the relation in Fig. 6 is unrelated to events within the membrane, and may depend on other factors, such as accumulation of cholesterol within the bulk phase torus surrounding the membrane. Evidently, a definitive answer to this issue must await a direct analysis of membrane composition.

It should be noted that there is considerable evidence, (summarized by Lampen [4] and Kinsky et al. [7]) that polyene antibiotics are bound directly to membrane-bound sterols. Furthermore, Lampen et al. (55) have shown that sterols may complex polyene antibiotics in aqueous solutions, and Kinsky et al. (7, 56) using negative staining techniques, have indicated that filipin may produce circular pits, approximately 125 A in diameter, in either mammalian erythrocyte membranes or cholesterol:lecithin dispersions. These workers have also noted that the lytic effects of polyene antibiotics on mammalian erythrocytes occur over a narrow concentration range (25).

If it is assumed that the interaction of cholesterol with nystatin or amphotericin B results in the formation of multimolecular membrane-bound units which increase membrane conductance, it is possible to make certain inferences concerning the effects of these units on the membranes. According to fixed charge theory (57), membrane permselectivity depends primarily on the net charge of fixed membrane sites. Since the membranes are predominantly anion-selective under these conditions (Tables II, IV, V), the interactions between nystatin or amphotericin B and membrane-bound cholesterol might reasonably be expected to result in membranes having a net positive charge. However, it is not apparent from the available information concerning the structure of these compounds ([2, 51, 52]; Fig. 8) how nystatin or amphotericin B can produce such a charge distribution. Furthermore, as shown in Fig. 5, when the other experimental conditions were kept constant, the relationship between the logarithm of membrane resistance and the logarithm of the salt concentration (in the range 0.0001–0.1 M) had a constant slope of approximately minus one. Thus, it is probable that under these conditions, the charge-carrying elements which traverse the membranes, regardless of their nature (see below), are units containing single ionic species (predominantly anions, but also cations) which move relatively independently of one another.

However, the experimental data are not adequate to determine whether the interaction of ions is directly with sites on cholesterol-polyene complexes within the membranes, or with phospholipids whose properties are modified by interaction with such aggregates. Although filipin (I.D. $\leq$ 4.5 A) did not lower membrane resistance, while the larger nystatin (estimated I.D. of the hypothetical model, Fig. 8B, $\approx$ 6.5–7.5 A) did, the later antibiotic did not discriminate between ions of different size, in the range 4.38–7.4 A diameter (Table III). Hence, an accomodation of ions into the inner diameter of the

larger polyene compounds (e.g., nystatin or amphotericin B) seems improbable. Furthermore, the experimental data are not adequate for evaluating whether the membrane sites responsible for the reduction in membrane resistance are mobile (i.e. carriers) or fixed (i.e., pores). In the former case, as indicated above, each carrier would probably involve a single ion (Fig. 5). However, if the sites directly responsible for the increases in the ionic conductances were composed of multimolecular aggregates of cholesterol and polyene antibiotic (nystatin or amphotericin B), it may be inferred that they are either fixed sites, or if mobile, present in high concentrations within the membrane phase, since the membrane conductances under these conditions are quite high (Table V), and the mobility of such a unit (cf. Equation 8) might reasonably be expected to be relatively low.

The pentaene filipin, which has a relatively small ring and lacks polar substituents, had no significant effect on the electrical properties of the membranes, but made them quite unstable (Table V). Hence, the antibiotic may have had little direct effect on the surfaces of the membranes, even at concentrations adequate for producing instability. The nature of the interactions of filipin with membranes which produce membrane instability are not understood, although it seems clear ([33]; Table V) that they are dependent on membrane-bound sterol. It has been suggested (6) that the effects of filipin are due to disruption of the hydrophobic interactions between CH_2 pairs in adjacent hydrocarbon chains of phospholipids.

Kinsky et al. (7, 33) have pointed out that saturation of the conjugated double bonds in the lactone ring results in polyene inactivation, at least with respect to filipin. Their observations suggest that the structural requirements for activity of the polyene antibiotics include, at a minimum, a cyclic, unsaturated lactone ring. A comparison of the effects of nystatin and amphotericin B (Tables II–V) with those of filipin (Table VI) on these thin lipid membranes suggests that the structural components of the polyene antibiotics responsible for the observed increases in ionic conductance (Table V) include a large number of ring atoms (approximately 36–37), the presence of either a free amino or a carboxyl group as a substituent in the molecule, or a combination of the three.

The applicability of Equation 1 in evaluating the ionic permeability of these membranes was restricted. As indicated in Fig. 7, the plot of V_m vs. the logarithm of the activity ratio of salt in the two aqueous phases was independent of the absolute salt concentration (in the range 0.0005–0.1 M). However, this relation deviated considerably from linearity when the activity ratio of NaCl in the two aqueous phases exceeded 25. Hence, under the latter conditions, other factors, in addition to differences in electrical potential or salt concentration between the two aqueous phases, contributed to the regulation of ion transport across the membranes. The participation of certain other

driving forces in ion transport could reasonably be excluded. Thus, the system was both isothermal and isobaric, and the magnitude of the membrane potential was independent of the direction of the ionic concentration gradient. The nonlinearity observed in Fig. 7 persisted when all of the aqueous phases were isotonic. Consequently, under these experimental conditions, solute-solvent interactions (i.e., "solvent-drag" [58]) did not substatially modify ion transport. In addition, the form of the relation in Fig. 5 provides reasonable evidence against the possibility of an appreciable degree of solute-solute interaction (59). The possibility that the rate of ion mixing in unstirred aqueous layers (60) adjacent to the membrane might contribute to the nonlinearity observed in Fig. 7 seems unlikely since the membrane conductances, although relatively high (Table V), were 10^{-3}–10^{-5} less than similar measurements for aqueous solutions. The possibility remains that the nonlinear relation observed in Fig. 7 is due to voltage-dependent changes in the ionic transference numbers, and studies to evaluate this issue are currently in progress.

The data presented in this paper are in accord with the observations of others (4, 7, 19, 23, 34) on the effects of these antibiotics on biological systems. To this extent, the interactions of the polyene antibiotics with thin lipid membranes may provide useful information for evaluating the effects of these agents on more complex systems, and may aid in understanding some of the mechanisms which regulate the transport of ions across membranes. In particular, the increases in the Na^+-dependent short circuit current observed when the toad bladder is exposed to amphotericin B (34) may be referable not only to a direct increase in the Na^+ permeability of the mucosal membrane (cf. Table V), but also because of a secondary, Cl^--related effect, due to an increased mucosal permeability to that anion, analogous to that observed in these membranes (Table IV). Finally, studies of the type described in this paper may be useful in the rational design of drugs which modify the ionic permeability properties of cellular membranes.

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