The Ion Permeability Induced in Thin Lipid Membranes by the Polyene Antibiotics Nystatin and Amphotericin B

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ABSTRACT Characteristics of nystatin and amphotericin B action on thin (<100 A) lipid membranes are: (*a*) micromolar amounts increase membrane conductance from 10^{-8} to over $10^{-2}\ \Omega^{-1}\ cm^{-2}$; (*b*) such membranes are (non-ideally) anion selective and discriminate among anions on the basis of size; (*c*) membrane sterol is required for action; (*d*) antibiotic presence on *both* sides of membrane strongly favors action; (*e*) conductance is proportional to a large power of antibiotic concentration; (*f*) conductance decreases $\sim 10^4$ times for a 10°C temperature rise; (*g*) kinetics of antibiotic action are also very temperature sensitive; (*h*) ion selectivity is pH independent between 3 and 10, but (*i*) activity is reversibly lost at high pH; (*j*) methyl ester derivatives are fully active; *N*-acetyl and *N*-succinyl derivatives are inactive; (*k*) current-voltage characteristic is nonlinear when membrane separates nonidentical salt solutions. These characteristics are contrasted with those of valinomycin. Observations (*a*)–(*g*) suggest that aggregates of polyene and sterol from opposite sides of the membrane interact to create aqueous pores; these pores are not static, but break up (melt) and reform continuously. Mechanism of anion selectivity is obscure. Observations (*h*)–(*j*) suggest—NH_3^+ is important for activity; it is probably not responsible for selectivity, particularly since four polyene antibiotics, each containing two —NH_3^+ groups, induce ideal *cation* selectivity. Possibly the many hydroxyl groups in nystatin and amphotericin B are responsible for anion selectivity. The effects of polyene antibiotics on thin lipid membranes are consistent with their action on biological membranes.

I. INTRODUCTION

The permeability properties of thin (<100 A) lipid membranes have received considerable attention since the technique for forming these structures was first described by Mueller et al. (1962 *a*, 1963). The conclusion of the studies on permeability to water, neutral solutes, and ions has been that these membranes display the characteristics expected of a thin layer having the low

dielectric constant and solvent properties of liquid hydrocarbons (Finkelstein and Cass, 1968). Matter crosses the membrane by dissolving in the hydrocarbon phase of the membrane and then diffusing through this region; the magnitude of the permeability coefficient for a given molecule can be at least semiquantitatively predicted from the solubility and diffusion coefficient of the molecule in the appropriate bulk hydrocarbon (Hanai and Haydon, 1966). Thus, these membranes are highly permeable to "lipophilic" solutes (Bean et al., 1968)[1] and even significantly permeable to water (Hanai and Haydon, 1966; Huang and Thompson, 1966; Cass and Finkelstein, 1967), but are very poorly permeable to "hydrophilic" solutes (Vreeman, 1966) and virtually impermeable to ions (Mueller et al., 1962 *b*; Vreeman, 1966). Because the thinness of these membranes is comparable with that of cell membranes and because they share with cell membranes major components such as phospholipids and sterols, they have enjoyed a certain notoriety as a model for biological membranes. However, the glaring discrepancy between the ion permeability of these films and that of natural membranes has stimulated attempts to modify these films to allow permeation by hydrophilic solutes, and more particularly by ions. In the search for modifying agents Mueller and Rudin have pioneered with their discoveries of "excitability inducing material, EIM" (Mueller and Rudin, 1968 *a*), and the cyclic peptide alamethicin (Mueller and Rudin, 1968 *b*), compounds which render the membranes cation permeable and electrically excitable, and of the cyclic depsipeptides, for example, valinomycin, which make the membranes both cation permeable and highly selective among the alkali ions though electrically inexcitable (Mueller and Rudin, 1967). In this and the following paper we discuss the action of another class of molecules, the polyene antibiotics, which greatly increase the membrane permeability to ions, water, and hydrophilic solutes.

The polyene antibiotics are a large class of antifungal agents produced by bacteria of the genus *Streptomycetes*. All the members of this class have a large polyhydroxylic lactone ring of 23–37 atoms with from 4 to 7 conjugated double bonds in the ring. Many polyenes have a carboxyl group and an amino sugar glycosidically linked to the ring (for a review of polyene chemistry, see Oroshnik and Mebane, 1963). The structures of two representative polyenes, filipin and nystatin, are shown in Fig. 1. Their biological activity appears to result from their ability to make cell membranes "leaky" to small solutes, and a large amount of evidence has accumulated to show that sterol is a necessary membrane constituent for their action. (For a review of polyene

[1] The values reported for the membrane permeability coefficients of such lipophilic molecules as indole and indole-3-ethanol are probably much too low, being essentially limited by the unstirred aqueous layers in series with the membrane. See the following paper (Holz and Finkelstein, 1970) for a discussion of this point.

biological activity, see Lampen (1966).) This sterol requirement extends to the action of polyenes on nonliving structures; thus, polyenes penetrate monolayers (Demel et al., 1965), disrupt phospholipid myelin figures (Kinsky et al., 1967), and destroy thin lipid membranes (van Zutphen et al., 1966), but only if these structures contain sterol.

In this paper we shall be particularly concerned with the effects of the polyene antibiotics, nystatin and amphotericin B,[2] on the ion permeability of thin lipid membranes; some data on filipin, pimaricin, PA-150, trichomycin, candicidin, and fungimycin will also be included. In the succeeding

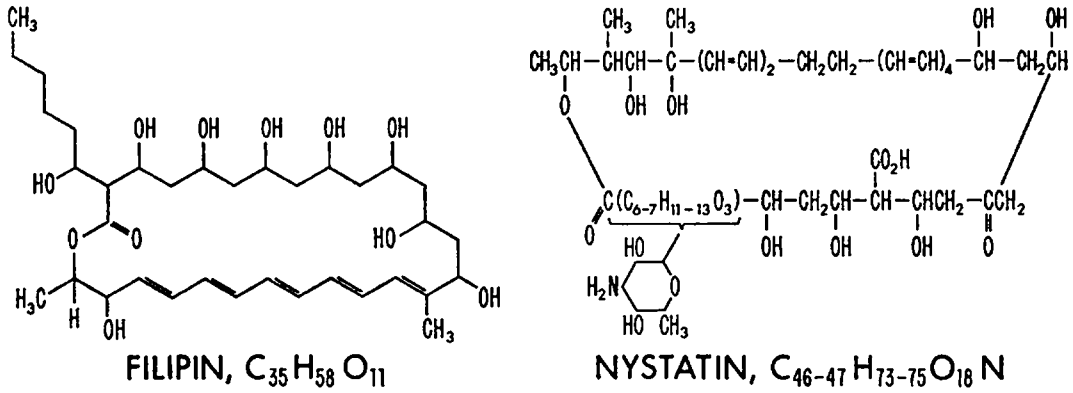

FIGURE 1. Formulas of nystatin and filipin.

paper we shall discuss the effects of nystatin and amphotericin B on water and nonelectrolyte permeability. We have reported some of these results previously (Finkelstein and Cass, 1968).

II. MATERIALS AND METHODS

Membranes were formed by the brush technique of Mueller et al. (1963). Unless otherwise stated, the lipids were deproteinized chloroform methanol extracts of ox-brain white matter (Mueller et al., 1963). 1 ml of a typical membrane-forming solution contained 2–4.4% lipid in 2:1 chloroform methanol, 200 mg *dl*-α-tocopherol, and 0–30 mg of additional cholesterol. Two experimental arrangements were used. In the first, membranes were formed across a hole (~1 mm^2) in a thinned sidewall of a polyethylene cup sitting in a Lucite dish. Mixing was accomplished in the cup by a magnetic flea and in the dish by bubbling with nitrogen. In the second arrangement, membranes were formed across a hole (~1 mm^2) in a polyethylene partition separating two Lucite chambers. Each side was stirred by a magnetic flea. This second arrangement was particularly useful for kinetic studies, because the combination of a thin partition (125 μ) and continuous stirring minimized the thickness of unstirred

[2] Amphotericin B differs from nystatin in that it contains an extra double bond between the diene and the tetraene, thus making the chromophore a heptaene. In other respects the molecules are structurally similar.

layers. In both arrangements the solutions on both sides were coupled to calomel electrodes through saturated KCl junctions, and the electrodes were in turn connected to a high-input impedance ($>10^{13}$ Ω) amplifier. For measurement of membrane resistance, a known step of current (ΔI) was passed through the electrodes, and the resulting steady potential difference (ΔV) across the membrane recorded through the same electrodes. The resistance was then given by $\Delta V/\Delta I$. The resistance of the electrodes ($\sim 2 \times 10^4$ Ω) was subtracted from the measured resistance when this was a significant correction, and for membranes of very low resistance, separate pairs of electrodes were used for passing current and recording potential. Salt gradients were established across the membrane by adding small amounts of concentrated salt solution to one side, and the resulting diffusion potential was recorded through the electrodes. The solutions on either side of the membrane could be completely changed by perfusing the given compartment with a new solution, the fluid level being maintained by suction.

The antibiotic being tested either was added drop by drop to both sides from a concentrated stock solution after the membrane had formed, or was added to the aqueous solutions prior to "painting" on the membrane, so that the membrane formed in the presence of the antibiotic. The stock solutions of nystatin, amphotericin B, filipin, and pimaricin were made up in methanol; those of PA-150, trichomycin, fungimycin, and candicidin were made up in either methanol or dimethylformamide; solutions were stored for months at −30°C without any significant loss of activity. Control experiments showed that methanol and dimethylformamide alone at concentrations in excess of the maximum used in the experiments were without effect. The nystatin used was either Squibb Mycostatin or a sample from batch HV-1793-A provided by Miss Barbara Stearns of the Squibb Institute for Medical Research; essentially the same results were obtained with each. Amphotericin B was a sample from batch 91368-001 also supplied by Miss Stearns.[3]

III. RESULTS

A. *Resistance of Membranes Treated with Nystatin or Amphotericin B*

1. THE GREATER EFFECTIVENESS OF NYSTATIN AND AMPHOTERICIN B WHEN PRESENT ON BOTH SIDES

In the absence of antibiotic, these membranes have resistances of the order 10^7–10^8 Ωcm² in 0.1 M salt solutions. When added to only one side of the membrane, nystatin and amphotericin B had rather irreproducible effects, even on successive membranes formed from the same membrane-forming solution. For example, nystatin concentrations of 10 μg/ml (≈10 μM) generally produced negligible changes in resistance. Yet on several membranes from the same membrane-forming solution, half this concentration reduced the resistance by a factor of 10^3 or more. Similar results were obtained with

[3] In our early experiments we used Squibb Fungizone, which is a water-soluble preparation of amphotericin B containing approximately equal weights of amphotericin B and deoxycholate. Our results with this preparation were comparable to those obtained with amphotericin B alone.

amphotericin B at concentrations of approximately 1 μg/ml (≈1 μM). In all instances, however, the further addition of a small amount of the antibiotic to the opposite side drastically lowered the resistance. When the antibiotic was added in equal concentrations to both sides, or when the membrane was formed in the presence of the antibiotic, reproducible results were obtained over a resistance range of six orders of magnitude. (At concentrations at which nystatin and amphotericin B in combination are ineffective from only one side, low resistance states are obtained when they are present on opposite sides.) Thus, the action of these antibiotics is strongly favored by their presence on both sides.

Whether the antibiotic is added to an already formed membrane or whether the membrane is formed in the presence of the antibiotic, a steady-state value of the resistance is generally achieved within 3 to 60 min, depending on temperature (see sections D and E). We may also note that when the membranes are formed in the presence of the antibiotic, the increase in conductance parallels the appearance of "black" regions. This demonstrates that the antibiotic action that we are observing is on the thin film.

2. DEPENDENCE OF MEMBRANE CONDUCTANCE ON ANTIBIOTIC CONCENTRATION

When nystatin is added in equal concentrations to both sides, the membrane conductance varies as a large power of the antibiotic concentration (Fig. 2 a). In a previous paper we stated that this was a tenth power relation (Finkelstein and Cass, 1968); we have subsequently found that this can vary with the lipid preparation, and the range to date is from 6 to 12 (see also Andreoli and Monahan, 1968 who report a 4.5 power relation with membranes formed from red cell lipid). We wish to emphasize, however, that the value of the exponent in the power relation is reproducible within 10% for membranes formed from any given membrane-forming solution.

We have found, generally, that the power relation for a given membrane-forming solution is lower for amphotericin B than for nystatin. Thus, when the power dependence is 10 for nystatin, it is about 7.5 for amphotericin B, and when 6 for nystatin it is about 4.5 for amphotericin B. For a representative membrane at 25°C, resistances of 10^2 Ωcm² in 0.1 M salt are obtained with nystatin concentrations of approximately 2–4 μg/ml and with amphotericin B concentrations of approximately 0.1–0.2 μg/ml. On this basis one would conclude that amphotericin B is the more potent agent. However, since the power relation is smaller for amphotericin B than for nystatin, the relative potency will depend on the conductance at which the two are compared, and in principle nystatin could eventually be termed more active than amphotericin B at very high conductances.

We have not observed any obvious correlation of the power relation with

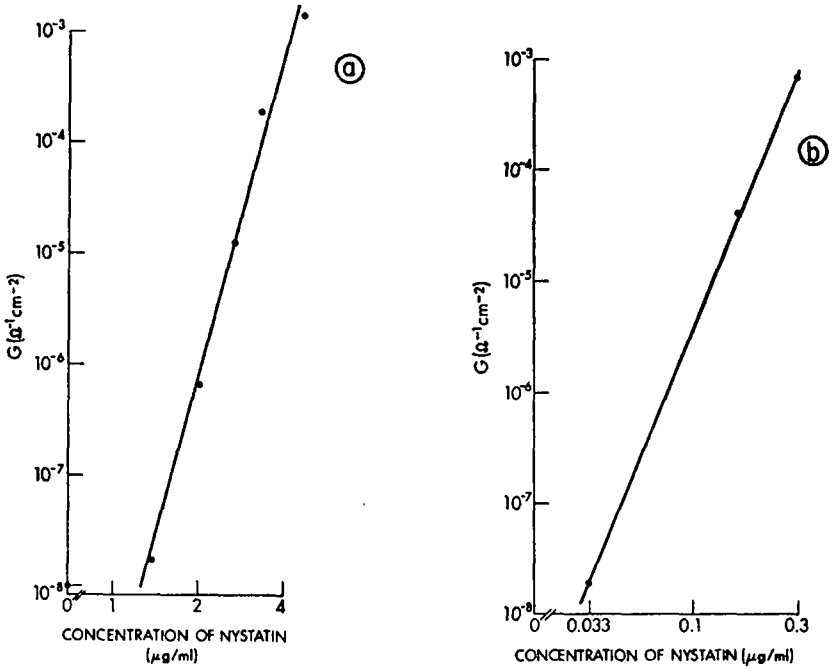

FIGURE 2 a. Conductance of a single thin lipid membrane as a function of nystatin concentration on both sides of the membrane. The membrane was formed in 100 mM NaCl, buffered to pH 7 with 5 mM sodium phosphate. After the membrane had formed, the concentration of nystatin was raised stepwise on both sides. The conductance reached a steady value within 20 min after each addition of nystatin. From the slope of the line we have $G \alpha C_{\text{nystatin}}^{9.6}$. Temperature = 29°C ± 0.5°C.

FIGURE 2 b. Conductance of a single thin lipid membrane as a function of nystatin concentration on one side with a large excess of nystatin on the opposite side. The membrane was formed in 100 mM NaCl buffered to pH 7 with 5 mM sodium phosphate. Membrane conductance after formation = $5 \times 10^{-9}\ \Omega^{-1}\ \text{cm}^{-2}$. Nystatin was added to one side to a concentration of 41 μg/ml. After 10 min the conductance reached a steady value of $2.8 \times 10^{-8}\ \Omega^{-1}\ \text{cm}^{-2}$. Nystatin was then added stepwise to the opposite side, and it is the concentration on this side that is plotted in the figure. (The first point plotted is the conductance minus the conductance with nystatin on only one side; namely $2.8 \times 10^{-8}\ \Omega^{-1}\ \text{cm}^{-2}$.) The conductance reached a steady value in approximately 45 min after each addition of nystatin. From the slope of the line we have $G \alpha C_{\text{nystatin}}^{4.8}$. This membrane was formed from the same membrane-forming solution used for the experiment in Fig. 2 a. Temperature = 26°C ± 0.5°C.

cholesterol content of the membrane-forming solution, though, as we noted, membranes are not sensitive in the absence of sterol (see also Andreoli and Monahan, 1968). Thus, membranes formed from a brain-lipid solution from which the cholesterol has been removed by passage through a Unisil (silicic acid) column showed only about a 20-fold resistance decrease at nystatin concentrations of 40 μg/ml. On the other hand, if cholesterol was added

back to this same cholesterol-depleted lipid at a phospholipid-cholesterol molar ratio of 1 : 1, the membrane resistance was lowered 10^5-fold with nystatin concentrations of only 2 μg/ml. Note that *dl*-α-tocopherol, present in the membrane-forming solutions, does not sensitize the membranes to the polyenes.

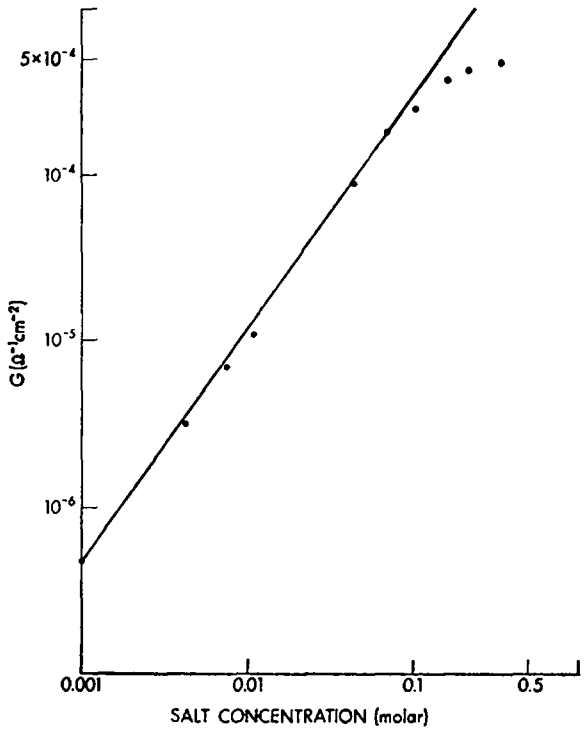

FIGURE 3. Conductance of a single thin lipid membrane treated with nystatin as a function of salt concentration. The membrane was formed in unbuffered 1.0 mM NaCl. After the membrane had formed, nystatin was added to both sides at a concentration of 1.33 μg/ml. After 40 min the membrane attained a steady conductance of 4.8×10^{-7} Ω^{-1} cm^{-2}. The NaCl concentration was then raised stepwise on both sides. The conductance reached a new steady level within 1 min after each addition of NaCl to both sides. From the slope of the line we obtain $G \alpha C_{NaCl}^{1.4}$. Temperature = 28°C ± 0.5°C.

For symmetrical addition of nystatin the exponent in the power relation for a given lipid is reproducible. If, however, nystatin is present in large excess on one side of the membrane, the conductance is found to vary as a smaller power of the concentration on the opposite side (Fig. 2 b). Thus, with symmetrical addition of nystatin to the two sides of the membrane, conductance is proportional to a 6th to 12th power of the antibiotic concentration. With a large excess of nystatin on one side of the membrane, however, conductance is proportional to approximately a 4.5 power of the antibiotic concentration on the opposite side. This same phenomenon is observed with amphotericin B.

In contrast to the large power dependence of conductance on antibiotic concentration, the conductance is *roughly* linearly dependent on the salt concentration between 0.001 M and 0.5 M (Fig. 3), a result also obtained by Andreoli and Monahan (1968).

B. *Ion Selectivity of Membranes Treated with Nystatin or Amphotericin B*

1. SINGLE SALT CASE

In the high conductance state produced by nystatin or amphotericin B, the membranes are anion selective. Fig. 4 is a plot of membrane potential as a function of the ratio of the salt activities on the two sides for the chloride salts of lithium, sodium, and potassium. Three points should be noted: First, the membranes are not ideally selective for Cl^-, the initial slope being ~48 mv instead of the ideal 59 mv. Second, the initial slope is essentially the same for each of the salts tested and is not dependent on the absolute concentration of salt used over the range 0.01 M to 0.1 M. Third, the plot is not a straight line but bends as the activity ratio increases; at larger absolute concentrations of salt this bending occurs for smaller activity ratios and is most pronounced for KCl, less so for NaCl, and least for LiCl and choline chloride. That is, the degree of bending is inversely related to the hydrated cation size. (This bending cannot be attributed to osmotic effects, as addition of sucrose to

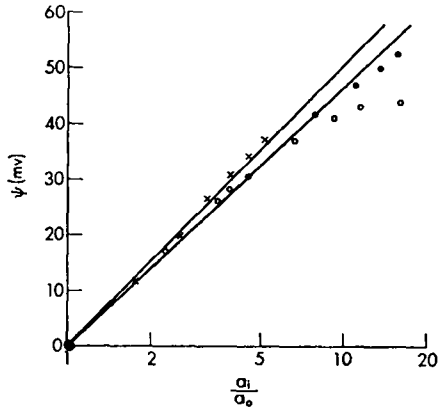

FIGURE 4. Potential difference (in millivolts) across thin lipid membranes treated with nystatin as a function of the ratio of activities of KCl (open circles), NaCl (solid circles), or LiCl (×) in the two aqueous phases. Membranes were formed in 10 mM solutions of the appropriate salt and nystatin was then added to both sides to a concentration of 1.6 μg/ml. Each membrane attained a conductance of approximately 10^{-4} Ω^{-1} cm^{-2}. Concentration differences were established across the membranes by addition of small amounts of concentrated salt solution to one side, and the resulting potential difference across the membrane was recorded. The slope, prior to bending, for KCl and NaCl is 47 mv; for LiCl the slope is 50 mv. Temperature = 26°C ± 1°C.

compensate for the differences in salt osmolarity has no effect on the potentials.) Single salt potentials with sodium isethionate give a smaller slope of approximately 35 mv.

The above results obtain when membrane resistance is between 10^6 and 30 Ωcm^2. For resistances much lower than 30 Ωcm^2 the single salt potentials decrease, and the membranes often break after several minutes. It appears that at these levels of resistance, large disruptions of membrane structure are occurring, with a consequent loss in selectivity and stability. (See Discussion for a comparison of this phenomenon with the results obtained with filipin.)

2. BI-IONIC CASE

On the basis of the single salt data, it appears that the membranes do not discriminate well between chloride and isethionate. This is not borne out, however, by the bi-ionic potentials of 60 mv obtained when the membrane separates 0.1 M NaCl from 0.1 M $NaSO_3CH_2CH_2OH$. In fact, on the basis of bi-ionic potential measurements, membranes treated with nystatin or amphotericin B appear to discriminate among anions on the basis of their hydrated size (Finkelstein and Cass, 1968). This discrimination is also reflected in conductance measurements. Thus with the same concentration of nystatin or amphotericin B, the conductance of membranes in 0.1 M NaCl is approximately eightfold greater than that of membranes in 0.1 M sodium isethionate. (The apparent discrepancy between these data and the single salt data will be commented on later.)

C. *Current-Voltage Characteristics in the Presence of Nystatin*

1. SINGLE SALT CASE

With identical concentrations of NaCl on the two sides of the membrane, the current-voltage (I–V) characteristic is linear between ± 75 mv. With gradients of NaCl, however, rectification is seen (Fig. 5); the conductance is smaller for positive currents than for negative currents.[4] The ratio of limiting slope conductances increases with the ratio of the concentrations. Within the limits of our time resolution the steady-state value of V for a given I is attained "instantaneously" (< 10 msec). Thus, there is no indication that the voltage dependence of the resistance is due to a time-variant change in the antibiotic-membrane interaction. Rather, the implication is that the voltage dependence is due to a shifting of the ionic profiles within the membrane. For a membrane 100 A thick this process would have a time constant of the order of 10^{-7} sec, and would be unmeasurable by our methods. The fact

[4] Positive currents are those for which current flows through the membrane from the more concentrated salt solution to the more dilute solution. The potential of the dilute solution is taken as 0.

that no rectification occurs with membranes separating solutions of the same NaCl concentration but different antibiotic concentrations is also consistent with this interpretation. The rectification in the single salt case will be discussed later.

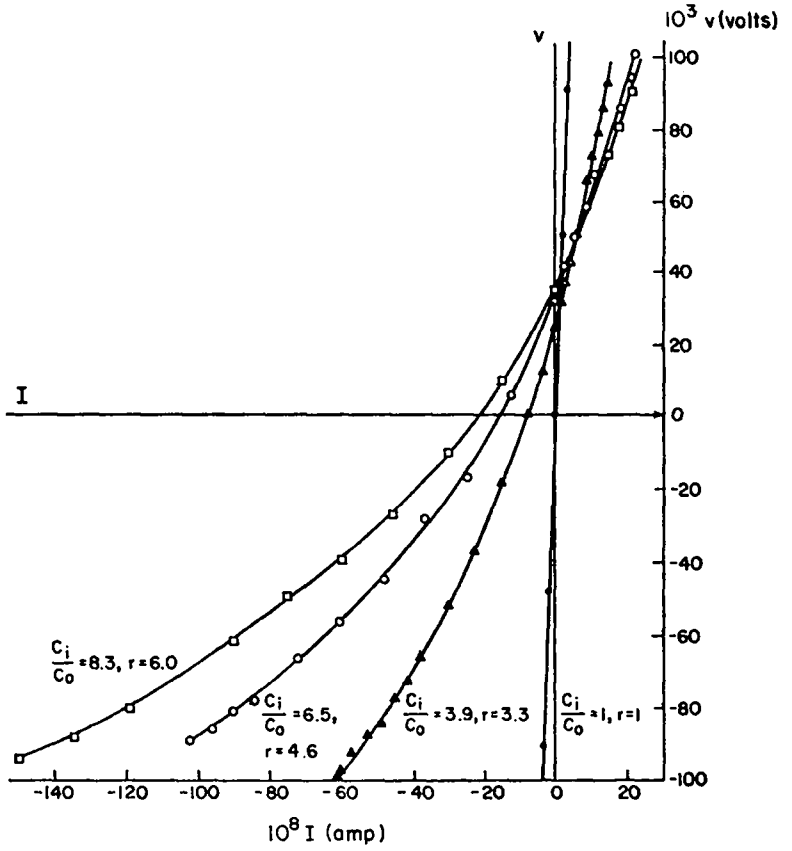

FIGURE 5. Current-voltage relations of a nystatin-treated thin lipid membrane in the presence of gradients of NaCl concentration with the same concentration of nystatin on the two sides of the membrane. r = the ratio of the limiting slope conductances; c_i/c_o = ratio of NaCl concentrations inside and outside. Initially, a membrane was formed with salt (100 mM) and nystatin (1.5 μg/ml) concentrations the same on both sides, and its curve is given by (solid circles). This membrane broke, and all the other curves are for a second membrane formed in the same solution, with sequential increases in the salt concentration ratio. Measurement of the steady-state potential developed during the passage of steps of constant current gave the curves plotted. Note the resting potentials due to the anion selectivity. Positive current is defined as passing from the more concentrated salt solution (inside) to the less concentrated solution (outside) and, accordingly, the potential V gives the potential of the inside with respect to the outside. With a given membrane, the rectification can be abolished by addition of salt to reduce the concentration ratio to 1; the current-voltage characteristic then becomes linear, having a slope consistent with the new higher level of salt concentration. Temperature = 23°C ± 0.5°C.

2. BI-IONIC CASE

The rectification curve obtained is qualitatively similar to that obtained in the single salt case (Finkelstein and Cass, 1968). The ratio of the limiting slope resistances of membranes separating 0.1 M NaCl from 0.1 M sodium isethionate is approximately the same as the ratio of the membrane resistances in the presence of 0.1 M solutions of each of the salts separately. The line of limiting slope for the sign of current producing the higher conductance extrapolates through the origin, whereas the line of limiting slope for the opposite sign fails to do so.

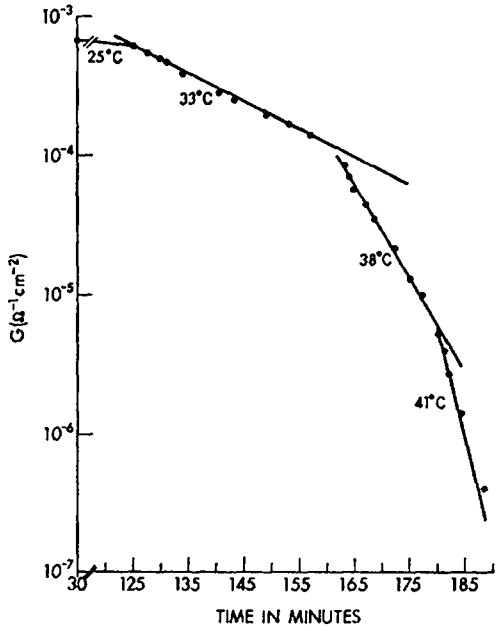

FIGURE 6. Kinetics of decrease of conductance of an amphotericin B–treated thin lipid membrane upon removal of the antibiotic from one side, and the effect of temperature on these kinetics. The membrane was formed at 25°C in 100 mM NaCl, buffered to pH 7 with 5 mM sodium phosphate. At zero time, amphotericin B was added to both sides to a concentration of 0.11 μg/ml. After 30 min the membrane conductance attained a value of $6.8 \times 10^{-4}\ \Omega^{-1}\ cm^{-2}$; at this time the solution on one side of the membrane was replaced by an amphotericin B–free solution and the conductance was followed in time. At the times corresponding to the breaks in the curve the temperature was raised to 33°, 38°, and 41°C. From the time required for the conductance to fall to $1/e$ of its previous value we obtain time constants of > 120 min, 22 min, 6.6 min, and 2.75 min for temperatures of 25°, 33°, 38°, and 41°C, respectively. The reciprocals of these times measure k_2 of equation (1). We see from these data that k_2 has a "Q_{10}" of approximately 10.

D. *Kinetics of Nystatin and Amphotericin B Action*

1. LOSS OF CONDUCTION SITES

When nystatin or amphotericin B is flushed out of one or both compartments, the conductance (g) decreases roughly exponentially with time (Fig. 6); that is

$$g = g_{eq.} e^{-k_2 t} + g_{\text{unmodified membrane}} \quad (1)$$

where k_2 is a constant and $g_{eq.}$ is the equilibrium conductance.

2. CREATION OF CONDUCTION SITES

When equal concentrations (p) of polyene (nystatin or amphotericin B) are added to both sides simultaneously, the conductance rises roughly exponentially (Fig. 7). The rate of rise of conductance for small times is proportional to the concentration raised to the α power, where α is the *same* power

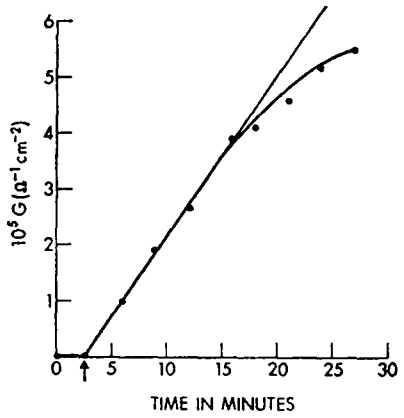

FIGURE 7. Kinetics of increase of conductance of a thin lipid membrane upon addition of equal concentrations of nystatin to both sides. The membrane was formed in 100 mM NaCl buffered to pH 7 with 5 mM sodium phosphate. Membrane conductance was $2 \times 10^{-8}\ \Omega^{-1}\ cm^{-2}$. At the arrow nystatin was added to both sides to a concentration of 2.5 μg/ml and the conductance was followed in time. From the initial slope we obtain a rate of rise of conductance of $0.275 \times 10^{-5}\ \Omega^{-1}\ cm^{-2}\ min^{-1}$. This is equal to $k_1 p^{\alpha}$ in equation (2). By repeating the experiment using a different concentration (p) of nystatin, k_1 and α are determined. With this particular membrane-forming solution α determined in this manner was 5.5, in excellent agreement with 5.85, the exponent obtained from the dependence of equilibrium conductance on nystatin concentration with membranes formed from this solution. Temperature = 26°C ± 0.5°C. Similar kinetic experiments performed on membranes from the lipid solution used in the experiment depicted in Fig. 2 a gave a value for α of 9.3, in excellent agreement with the exponent of 9.6 in the equilibrium experiment shown in Fig. 2 a.

as that determining the dependence of the equilibrium conductance on concentration. That is,

$$g = \frac{k_1}{k_2} p^{\alpha}(1 - e^{-k_2 t}) + g_{\text{unmodified membrane}} \quad (2)$$

where k_1 is a constant. If a large excess of antibiotic is first introduced on one side and then sometime later a small amount added to the opposite side, the kinetics of conductance rise are those described by equation (2), except that α is replaced by approximately $\alpha/2$. We have noted the analogous change in the power dependence for the equilibrium situation (compare Figs. 2 a and 2 b). The general kinetic equation with equal concentrations on both sides is given by:

$$\frac{dg}{dt} = k_1 p^{\alpha} - k_2 g. \quad (3)$$

E. *The Effect of Temperature*

In the presence of nystatin the membrane conductance is extraordinarily sensitive to temperature, the conductance decreasing with increasing temperature (Finkelstein and Cass, 1968). So-called Q_{10} values of the order of 10^4 are obtained.[5]

In addition, the kinetics of creation and loss of conduction sites are also very sensitive to temperature. The effect of temperature on the constant k_2 in equation (3) is illustrated in two different types of experiments. First, the time rate of decrease in conductance as the temperature is raised rapidly from say 28° to 33°C and then held at 33°C is much lower than the rate obtained when the temperature is raised from 33° to 38°C and then held at 38°C.

Second, the rate at which conductance falls when nystatin or amphotericin B is flushed out of one or both solutions is also critically dependent on temperature (Fig. 6). The Q_{10} of k_2 is approximately 10 for amphotericin B and 5 for nystatin. At 25°C, k_2 is much smaller for amphotericin B than for nystatin. For example, with membranes formed from one particular lipid solution, there was no significant decrease in conductance 1 hr after amphotericin B was flushed out of solution, whereas conductance decreased with a half-time of 20 min when nystatin was removed from solution.

With nystatin the effect of temperature on the constant k_1 in equation (3) is of greater magnitude than the effect on k_2.[6] The rate of rise of conductance

[5] The conductance of an amphotericin B–treated membrane can also be greatly lowered by raising the temperature, but the changes due to temperature are poorly reversible.

[6] Although the "on" kinetics with amphotericin B at about 25°C are comparable to the kinetics with nystatin, they are more complicated at elevated temperatures and will not be discussed here.

when nystatin is added to both sides is about 1000-fold smaller at 36°C than at 26°C. (We are referring to the absolute rate of conductance change in Ω^{-1} min^{-1} and *not* to the *time constant* of this change, which, being determined by k_2, is faster at the higher temperatures. We must recall that for a given concentration of nystatin, the final conductance is about 10^4 times smaller at 36°C than at 26°C.) Setting $\frac{dg}{dt} = 0$ in equation (3), we have for the equilibrium conductance:

$$g_{eq.} = \frac{k_1 p^\alpha}{k_2} \tag{4}$$

It appears, therefore, that the decrease in equilibrium conductance with temperature is due to both an *increase* of the destruction rate constant, k_2, and a *decrease* in the formation rate constant, k_1, with the major effect being that of a decrease in k_1.

F. *The Effect of pH on Ion Selectivity and Resistance*

Since nystatin and amphotericin B each have one carboxyl and one amino group, it is possible that the anion selectivity these molecules induce is a consequence of these charged groups. In order to assess this possibility, we have examined as a function of pH the single salt potentials obtained with NaCl in a nystatin-treated membrane. We found no change in these potentials between pH 3 and 10. Since the carboxyl group is presumably being titrated in the low pH range, it is unlikely that the selectivity is controlled by this charged group. However, we have found that if the pH is raised above 9.5, the membrane resistance increases; and at approximately pH 10.5 nystatin has virtually no effect on membrane resistance. This effect of high pH is reversible, the membrane resistance falling to the usual values in nystatin or amphotericin B solutions when the pH is brought back to 8 or lower.[7] Since above pH 9.5 the $-NH_3^+$ is probably being titrated to $-NH_2$, it appears that the ionized form is important for the interaction of nystatin or amphotericin B with the membrane. Since we have been unable to find conditions in which the resistance remains low even though the amino group should have been completely titrated to the neutral form, we cannot rule out that the $-NH_3^+$ contributes to the ion selectivity. At a given temperature the rate of increase in resistance produced by raising the pH to 10.5 is much faster than that produced by flushing nystatin or amphotericin B out of the system at pH 7, so that the pH effect must be directly on polyene that has already interacted with the membrane.

[7] If the solutions are left for some time at high pH, the effect is irreversible. This is probably due to inactivation of polyene in solution by base-catalyzed hydrolysis of the lactone ring.

G. *Derivatives of Nystatin and Amphotericin B*[8]

In order to study further the role of the amino and carboxyl groups in the polyene membrane interaction, we examined the activity of nystatin-methyl ester, amphotericin B–methyl ester, *N*-acetyl nystatin, and *N*-succinyl nystatin. We found no loss of activity or increase in selectivity with the methyl esters but approximately a 90% loss of activity with the *N*-acetyl and *N*-succinyl derivatives, and, in fact, the residual activity may have been due to unreacted nystatin. This result is in agreement with the loss in biological activity of polyene antibiotics when they are *N*-acetylated (Lampen, 1966). Since the *N*-acetyl and *N*-succinyl derivatives have a net negative charge, it is possible that their inactivity is the result of electrostatic repulsion between polyene and negative charges on the membrane. In order to test this we checked the activity of nystatin that had been both *N*-acetylated and methyl-esterified. This derivative was also inactive, although, as mentioned above, the methyl ester derivative itself was fully active. These results, taken together with the effect of pH described above, imply that the $-NH_3^+$ group is necessary for nystatin or amphotericin B to interact with the membrane, whereas the carboxyl group is not particularly significant for this interaction or the selectivity. We have found, however, that the k_2's for the methyl ester derivatives are much larger (at least by a factor of 10) than the k_2's for the parent compound. Thus, at 25°C, removal of the methyl ester derivative from solution causes the membrane conductance to decrease with a half-time of approximately 1 min, whereas the half-time for nystatin is 20 min and for amphotericin B is well over 2 hr. We may also mention that perhydronystatin (all carbon=carbon double bonds hydrogenated) is virtually completely inactive,[9] suggesting that the rigid conformation of the ring is required for function.

DISCUSSION

A. *The Meaning of the Dependence of Conductance on a Large Power of the Nystatin or Amphotericin B Concentration*

The most obvious meaning of the exponent, α, in equation (4) is that it represents the number of polyene molecules that have interacted with the membrane to form a conduction site. We have noted that α can be anything from 4 to 12, depending on the membrane lipid. Yet in terms of ion selectivity and nonelectrolyte permeability there are no significant differences between a nystatin- or amphotericin B–treated membrane that follows a low power

[8] The derivatives discussed here were all synthesized by Dr. Robert Yu. A detailed report on the synthesis and purity of these samples will be presented elsewhere.

[9] At large concentrations perhydronystatin breaks the membranes.

dependence and one that follows a high power dependence. If α represents the number of polyene molecules forming a site, it is surprising that a conduction site made up of 4 nystatin molecules should have the same permeability as one consisting of 12 molecules. This invariance of permeability with α makes us somewhat uneasy about our interpretation of the meaning of α, but we have not been able to devise a viable alternative to it. The kinetics suggest that α polyene molecules, or rather approximately $\alpha/2$ from each side of the membrane, interact to form a site.

A picture that appears consistent with the data available for nystatin and amphotericin B is the following: Monomers of polyene (p) in solution are in equilibrium with aggregates (a) on each side of the membrane consisting of $\alpha/2$ polyene molecules and an unknown number (x) of sterol molecules (s). That is, at each interface:

$$\frac{\alpha}{2} p + xs \rightleftarrows a \tag{5}$$

If we assume that the number of sites on the membrane is in great excess, then,

$$(a) = K(p)^{\alpha/2} \tag{6}$$

where K is an effective equilibrium constant. When aggregates from opposite sides overlap sufficiently, they combine to form a conduction site, g. That is:

$$a \text{ (side 1)} + a \text{ (side 2)} \underset{k_2}{\overset{k_1'}{\rightleftarrows}} g \tag{7}$$

At equilibrium, we have by combining (7) and (6) and assuming equal concentrations of polyene on the two sides:

$$g_{\text{eq.}} = \frac{k_1'}{k_2} K^2 (p)^{\alpha} \tag{8}$$

Thus, the conductance is proportional to the polyene concentration raised to the α power. Comparing equations (8) and (4) we have

$$k_1 = k_1' K^2 = k_1' \frac{(a)^2}{(p)^{\alpha}} \tag{9}$$

The formation rate constant k_1 is seen to be the product of a rate constant k_1' for the formation of conduction sites from aggregates on opposite sides, and an effective equilibrium constant K for the formation of these aggregates from polyene molecules in solution and membrane sterol.

For simplicity we have assumed in our derivation that *monomer* in solution

interacts with the membrane. It is possible, however, that there exists in solution the equilibrium:

$$\frac{\alpha}{2} p \rightleftarrows p_{\alpha/2} \tag{10}$$

and that the $p_{\alpha/2}$ species interacts with the membrane. Provided that $(p_{\alpha/2}) \ll (p)$, equations (5) through (9) still hold. Of course, the effective equilibrium constant K will then include the equilibrium constant for the reaction depicted in (10).

In this picture the large temperature dependence of the formation kinetics (k_1) must be attributed to the temperature dependence of the reaction in (5);[10] that is, to the temperature dependence of the equilibrium constant K. (It is almost inconceivable that a true rate constant, such as k_1', should decrease with increase in temperature.) Thus, with increasing temperature the aggregates melt; since at a given polyene concentration k_1 is proportional to the concentration of aggregates, this will be reflected as a decrease in the formation rate. The temperature dependence of the destruction kinetics (k_2) is probably a reflection of the large energy of activation associated with break up of the conduction site.[11]

In this model we have assumed that at all times equilibrium exists between polyenes in solution and the aggregates (or "half-conduction sites") on each side of the membrane; that is, we assume that the rate of this reaction is large compared to the rate of combination of aggregates from each side to form a conduction site. This assumption is supported by the following two observations: First, if at a time after polyene has been added to both sides and while conductance is still increasing, the polyene is flushed out from one side, the conductance stops increasing. This indicates that polyene on or in the membrane on a given side but not participating in a conduction site is very swiftly removed if polyene is flushed from the aqueous phase. Second, if polyene which has been in contact with one side for an extensive time is flushed out and polyene then added within 3 min to the opposite side, there is no increase in membrane conductance. This, despite the fact that at the same temperature (25°C) the disappearance of conduction sites has a half-time of 20 min for nystatin and over 2 hr for amphotericin B. Thus, half-conduction sites have a much shorter life time than conduction sites.

B. *The Nature of Conduction Sites Created by Nystatin or Amphotericin B*

The data on the permeability of nystatin- and amphotericin B–treated membranes to water and nonelectrolytes offer strong evidence that these polyenes

[10] If the reaction in (10) is assumed to occur, then the temperature dependence of k_1 might also be attributed to the temperature dependence of this reaction.

[11] In this analysis we have neglected the possible dependence of α on temperature.

form aqueous pores in the membrane (Holz and Finkelstein, 1970). Also suggestive of pore formation is the apparent multimolecularity of the reaction of these polyenes with the membrane and the greater effectiveness of these agents when present on both sides of the membrane. In the following paper we discuss the so-called "equivalent radius" of these pores; at this point it is sufficient to note that the nystatin- and amphotericin B–treated membranes are essentially impermeable to glucose, a molecule whose Stokes-Einstein radius is approximately 4 A. With regard to pore size, however, it is instructive to compare the action of nystatin and amphotericin B with the action of filipin. Electron micrographs of negatively stained red cell membranes and liposomes that have interacted with filipin reveal "pits" of radii greater than 100 A (Kinsky et al., 1967). We have found that filipin (at 5 μg/ml on one side) causes breakage of thin lipid membranes at relatively high resistances,[12] and we have been notably unsuccessful in obtaining stable filipin-treated membranes having resistances less than 10^6 Ωcm^2. (This has also been the experience of other investigators [Andreoli and Monohan, 1968].) During the evanescent low-resistance states which are sometimes obtained, the membranes exhibit poor ion selectivity, as evidenced by negligible diffusion potentials with NaCl gradients. The instability and the poor ion selectivity are consistent with filipin creating large pores or pits in thin films, as it apparently does in liposomes and red cell membranes. This is to be contrasted with the effects of nystatin and amphotericin B, which leave the thin film stable with significant ion selectivity at resistances as low as 30 Ωcm^2. We have mentioned, however, that at large concentrations even these antibiotics will drive the membranes into a state of poor ion selectivity and instability. It is possible that at these large concentrations, large pores are formed (perhaps by confluence of the small pores), causing such membranes to resemble filipin-treated films.

C. *Structure of the Pores*

If one looks at a space-filling model of nystatin, one is immediately seduced by the hole in the ring; and indeed, the hole size is roughly consistent with the selectivity observed among small water-soluble solutes. It is, therefore, tempting to speculate that pores are formed by the stacking of nystatin molecules through the membrane. There is at present, however, no compelling evidence to support this picture. Furthermore, if one assumes that the polyenes share a common mode of action on these membranes, then it is quite clear that the pore is not formed by the hole in the antibiotic, since the small-ringed filipin molecule creates much larger pores than the large-ringed nystatin and amphotericin B.

A more likely mechanism is that several polyene molecules upon interacting

[12] Pimaricin also breaks thin lipid membranes at high resistances.

with several cholesterol molecules of the film produce a structural rearrangement (micellization?) of a region of the bilayer, thus creating a pore. (A similar suggestion has been made by Demel et al. (1965) based on monolayer studies.) The entrance to the pore might contain a polyene, and in this way the nystatin and amphotericin B ring could directly affect selectivity. From our data on the two-sided effect of nystatin and amphotericin B and the smaller power relation obtained when an excess of antibiotic is on one side and small amounts are added to the opposite side as compared with the power relation obtained when antibiotic is added symmetrically to both sides, it appears that this rearrangement in structure can occur quasi-independently in the two halves of the bilayer. That is, "half-pores" are formed at each interface, and when two such half-pores from opposite sides overlap sufficiently, a complete pore is formed. The mechanism by which interaction of polyene and cholesterol produces the structural reorganization is at present not understood.

It is interesting to note that the pores do not appear to be static or permanent, since the membrane resistance rises to its original high value upon removal of nystatin or amphotericin B from one or both sides. Therefore, at equilibrium, with polyene in the aqueous solutions, pores must be forming and unforming all the time. As we have seen from the large temperature dependence of the kinetics, the rates of these processes can vary tremendously, so that at low temperatures the pores have a more "permanent" character than at high temperatures. This dynamic aspect of nystatin- and amphotericin B–created pores is perhaps relevant to the idea mooted in the literature that biological membranes are dynamic structures undergoing continuous change and that lipid structures may readily transform from a bilayer to a micellar state (Lucy, 1964).

D. *Density of Pores*

Up to this point we have not considered the area occupied by the nystatin- or amphotericin B–created pores and have perhaps given the erroneous impression that a large fraction of the membrane area has been transformed into a porous structure. This, however, is probably not the case. As is shown from the tagged water permeability data, a membrane whose resistance in 0.1 M NaCl has been brought down to the low value of 100 Ωcm^2 with nystatin has less than 0.01% of its area occupied by pores (Holz and Finkelstein, 1970). These membranes must, therefore, be mosaic structures consisting primarily of lipophilic unmodified regions with occasional aqueous pores sprinkled throughout.

This mosaic aspect has been dramatized by treating a membrane with both nystatin and valinomycin (Finkelstein and Cass, 1968). We showed that the membrane could be made either anion- or cation-selective, depending

on the relative amounts of nystatin and valinomycin added; the two molecules appear to act independently and in parallel, an action consistent with the view that there is plenty of room available for valinomycin in the nystatin-treated membrane. This independence was further emphasized by our ability to utilize the high temperature coefficient of the nystatin regions to shift reversibly between anion and cation permeable states simply by raising and lowering the temperature a few degrees.

E. *Comparison of the Effects of Nystatin and Amphotericin B with Those of Valinomycin*

Much attention has been given to the effects of the depsipeptides, particularly valinomycin, on thin lipid membranes (Mueller and Rudin, 1967; Lev and Buzhinski, 1967; Andreoli et al., 1967). It is interesting to compare the nature and action of these molecules with those of the polyenes. In Table I we draw such a comparison between nystatin and valinomycin. Whereas the conductance increases as a large power of the nystatin concentration, it is simply linearly dependent on the valinomycin concentration. This linear dependence suggests that valinomycin acts as a mobile carrier of ions. There are additional reasons for believing this. First, it can be demonstrated that valinomycin crosses the membrane. This is seen by stirring either on the side to which valinomycin has been added or on the opposite side. In the former instance the conductance rises, in the latter instance it falls. Both results indicate that valinomycin crosses the membrane, the stirring perturbing the boundary concentrations. Second, it has been shown that valinomycin can carry K^+ into a bulk organic phase (Pressman et al., 1967), and we may assume that

TABLE I

COMPARISON OF VALINOMYCIN AND NYSTATIN

	Valinomycin	*Nystatin*
Chemical structure	Cyclical depsipeptide; many carbonyl groups (C=O)	Cyclical polyenic lactone; many hydroxyl groups (C—OH)
Solubility in hydrocarbon	Soluble	Insoluble
Ion selectivity produced in membrane	Cation	Anion
Specific lipid required in membrane for action	None	Sterol
Dependence of membrane conductance (g) on antibiotic concentration (c)	g proportional to c	g proportional to c^α, $\alpha > 4$
Is action on membrane potentiated by presence on both sides?	No	Yes
Effect of temperature on membrane conductance	g increases with temperature; $Q_{10} \approx 8$	g decreases with temperature; $Q_{10} \approx 10^4$

it can also carry K^+ into the hydrocarbon region of the membrane. In fact, aside from the details of the chemistry, the most striking over-all difference between the depsipeptides and the polyenes is that the former have a hydrocarbon exterior, thus making them quite soluble in hydrocarbon, whereas the polyenes are amphipathic molecules that are totally insoluble in hydrocarbon. This latter fact makes it unlikely a priori that nystatin and amphotericin B would act as carriers. We have previously discussed in connection with the iodine-iodide system how the delocalizing of charge over a larger surface area can strikingly increase the solubility of an ion in a low dielectric constant medium (Finkelstein and Cass, 1968). Presumably, this is the mechanism operating also in the case of valinomycin and potassium. That is, with K^+ buried within the complex the effective size of the ion has increased, making the valinomycin-K^+ complex many orders of magnitude more soluble in hydrocarbon than K^+ itself. Third, that cholesterol-containing membranes in the presence of valinomycin have a lower conductance than do cholesterol-free membranes (Mueller and Rudin, 1967; personal observations) is also consistent with valinomycin being a mobile carrier. For, if as has been suggested, the lower water permeability of cholesterol-containing membranes is due to a greater "viscosity" of the hydrocarbon phase and hence a lower mobility of water in this phase (Finkelstein and Cass, 1967), this same effect of cholesterol should be operative on any molecule that diffuses through the hydrocarbon region. That is, the mobility of a molecule, such as the valinomycin-K^+ complex, diffusing through the hydrocarbon region would be smaller in a cholesterol-containing membrane than in one that was cholesterol-free. It is amusing that a membrane treated with both nystatin and valinomycin has two mechanisms for ion movement often attributed to cell membranes, namely, aqueous pores and mobile carriers.

F. *Ion Selectivity and Current-Voltage Relations*

1. MECHANISM OF SELECTIVITY

From the potential measurements in the single salt case, we have seen that in the presence of nystatin or amphotericin B, the membranes are more permeable to anions than to cations, although they are not completely selective for anions. The question arises as to the mechanism for this selectivity. As we have shown, the selectivity is unaffected by changes of pH between 3 and 10, and over this range the carboxyl and amino groups should be significantly titrated. Furthermore, we have observed the same anion selectivity in membranes formed from ox-brain lipids and cholesterol as in those formed from monophosphoinositide and cholesterol; in the former the phospholipids have both negative and positive charged groups, but in the latter the phospholipid has only negative charged groups. It appears, therefore, that selectivity cannot be attributed, in any obvious way, to the two charge

groups on the polyene or to the charges on the phospholipids. The only other likely candidates are the many hydroxyl groups on the polyenes. Just as the carbonyl groups on the depsipeptides have been invoked to explain the cation selectivity of the depsipeptides (Mueller and Rudin, 1967), one can similarly argue that the hydroxyl groups are able to substitute for the outer hydration shells of anions. Thus, the energy necessary for transferring an anion from the surrounding solution into the pore would be less than the energy necessary for transferring a cation. One may picture that the interior of the pore is lined by hydroxyl groups (or that the pore entrance is controlled by a polyene ring).

We should probably let well enough alone and defer further discussion of this selectivity problem. Unfortunately, we must report that four other polyene antibiotics, candicidin, fungimycin (perimycin), trichomycin, and PA-150 make these membranes ideally *cation* selective as determined from single salt diffusion potentials with NaCl and KCl. A further confusion is that candicidin has two amino groups and one carboxyl group, and fungimycin has two amino groups and no carboxyl groups; that is, candicidin has a net charge of $+1$ and fungimycin a net charge of $+2$. Both compounds are rather large polyenes whose structures have only been partially worked out. Hopefully, there are some hidden aspects of their structures to redeem the situation. All four of these polyenes are as effective from one side as from two, in contrast to the behavior of nystatin and amphotericin B. In addition, the conductance of a membrane separating identical salt solutions with either candicidin or fungimycin on one side is dramatically voltage dependent, displaying a region of negative slope resistance. A full description of these findings will appear in a future publication.

Turning from the basis of the anion selectivity to the selectivity *among* anions, we find a less confusing situation. From both the bi-ionic potential measurements and the single salt conductances, it appears that selectivity is based on hydrated ion size. We mentioned previously that the selectivity among anions is more clearly revealed by bi-ionic potential measurements than by comparison of single salt potentials. This is probably because the transmembrane potential can be viewed at least qualitatively as the sum of two boundary potentials arising from the partition coefficients of the ions, plus an interior diffusion potential. In the single salt case, the boundary potentials tend to cancel, whereas these are fully manifest in the bi-ionic case. It should be realized, however, that in dealing with a membrane of <100 A thickness it is really not possible to split the membrane potential in this way, as the space-charge regions will extend throughout the membrane.

2. CURRENT-VOLTAGE CHARACTERISTIC OF SINGLE SALT CASE

Since we have indicated that fixed charges probably do not determine the ion permeability, the rectification observed in the single salt case (see Fig. 5)

is somewhat unexpected; for, as is well-known, a Planck diffusion regime for the single salt case behaves as a simple ohmic resistance (Planck, 1890). Again, we feel that the rectification results from the failure of this system to satisfy the electroneutrality condition, because of the thinness of the membrane, and that the voltage dependence of the resistance results from perturbation of the space charge regions by the applied field. This voltage dependence, the magnitude of the single salt and bi-ionic potentials, and the bending of the potential vs. activity ratio curve (see Fig. 4) will hopefully fall out as analytical consequences of the combination of the flux equations and Poisson's equation with appropriate boundary conditions. Such an analysis is presently in progress.

G. *Comparison of the Action of Polyene Antibiotics on Thin Lipid Films with Their Action on Biological Membranes*

The action of polyenes on thin lipid films is both qualitatively and quantitatively consistent with what is known of their effects on plasma membranes. The agreement extends to sterol requirement, severity of damage to the membrane (filipin > amphotericin B and nystatin), absolute concentrations required, loss of activity with *N*-acetylation and hydrogenation, and the making of the membrane leaky to small solutes. In fact, their lytic effect on cells probably corresponds to that state of the films, achieved at relatively high antibiotic concentrations, in which selectivity is lost and membranes break. Their sublytic effects on cell metabolism (Lampen, 1966), on the other hand, probably correspond to less drastic alterations in the membrane permeability, corresponding to the more subtle permeability changes observed in the films. It is unfortunate that the quantitative data obtained with the artificial membranes are not yet available for biological membranes; in particular, it would be of interest to establish whether nystatin and amphotericin B produce anion selectivity in biological membranes and whether candicidin, fungimycin, trichomycin, and PA-150 produce cation selectivity. It is also of interest that we have found that thin lipid membranes containing ergosterol respond to nystatin at much lower concentrations than do those containing cholesterol. This may explain why it is possible to use polyene antibiotics therapeutically in the treatment of internal fungal infections; that is, a concentration range exists that is fatal to the ergosterol-containing fungal membrane but is relatively ineffective against cholesterol-containing mammalian cells. In summary, although there are undoubtedly many differences between plasma membranes and thin lipid membranes, the data presently available indicate that these are not significant in determining the effects produced by polyene antibiotics.

We thank Dr. C. M. Connelly for his painstaking reading of the manuscript.
This work was supported by U.S. Public Health Service Grant NB 03356 and Grant No. 14-01-0001-1277 from the Office of Saline Water, U.S. Department of the Interior.
Dr. Finkelstein is a Career Development Awardee of the U.S. Public Health Service.

Received for publication 7 January 1970.

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