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Single-Length and Double-Length Channels Formed by Nystatin in Lipid Bilayer Membranes

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Summary. Nystatin forms two types of channels in sterol-containing planar bilayer membranes. One type is formed when it is added to only *one side* of the membrane; the other is formed when it is added to *both sides* of the membrane. The relative permeability of these channels to nonelectrolytes (urea and glycerol) is identical. The sensitivity of membranes to the one-sided action of nystatin is critically dependent on their thickness; in particular, membranes made from monoglycerides with more than 18 carbon atoms in their acyl chain are insensitive to nystatin's one-sided action. These data are consistent with a model in which the two types of channels formed by nystatin have essentially identical structures, except that the channel formed by its two-sided action is twice the length of that formed by its one-sided action, because it is a tail-to-tail dimer of the latter.

Key Words polyene antibiotics · single channels · ion selectivity · nonelectrolyte permeability · lipid bilayers · bilayer thicknesses

Introduction

When added to *both sides* of sterol-containing planar lipid bilayer membranes, the anti-fungal polyene antibiotics nystatin and amphotericin B (Fig. 1) increase membrane permeability both to univalent ions (anions more so than cations) and to water and small nonelectrolytes (Andreoli & Monahan, 1968; Andreoli, Dennis & Weigl, 1969; Cass, Finkelstein & Krespi, 1970; Holz & Finkelstein, 1970). The permeability coefficients for nonelectrolytes decrease monotonically with increasing molecular size and are immeasurably small for molecules larger than glucose. These "sieving" data are most easily explained by the assumption that nystatin and amphotericin B form aqueous channels of approximately 4 Å radius in lipid bilayers (Holz & Finkelstein, 1970), and a space-filling (CPK) molecular model of amphotericin B suggests that the channel is formed by two "barrels" hydrogen-bonded end to end, with each barrel consisting of 8 to 10 amphotericin B (nystatin) monomers arranged circumferentially as staves (Fig. 2) (Finkelstein & Holz, 1973; de Kruijff & Demel, 1974).

When added to only *one side* of sterol-containing planar lipid bilayer membranes, nystatin and amphotericin B again increase membrane permeability to univalent ions, but in this instance the permeability is more to cations than to anions (Marty & Finkelstein, 1975). Since the nonelectrolyte permeability induced by the one-sided action of nystatin and amphotericin B on plasma membranes is consistent with that induced by its two-sided action on planar bilayers (Cass & Dalmark, 1973; de Kruijff et al., 1974), Marty & Finkelstein (1975) proposed that the one-sided action on both plasma membranes and planar bilayers was attributable to channels having essentially the same structure as those formed by the two-sided action, except that only one "barrel" (instead of two barrels hydrogen-bonded end to end) spanned the bilayer to form a functional channel. They argued that enough flexibility exists in bilayer thickness for the same bilayer to accommodate both of these channels, even though they differ in length by a factor of two (Fig. 3).

The above proposal for the existence and structure of "single-length" channels, and their relationship to "double-length" channels, has three experimentally testable implications: (1) Both the single-length and double-length channels are formed by the same molecule (nystatin or amphotericin B); that is, even though much larger concentrations are required for the one-sided than for the two-sided effect (Marty & Finkelstein, 1975) and Squibb nystatin is only about 80% pure (Mechlinski & Schaffner, 1974), a contaminant molecule is not responsible for the one-sided effect. (2) If channels formed by the single-sided action of nystatin and amphotericin B are only half the length of those formed by their two-sided action, a maximum bilayer thickness should exist beyond which nystatin

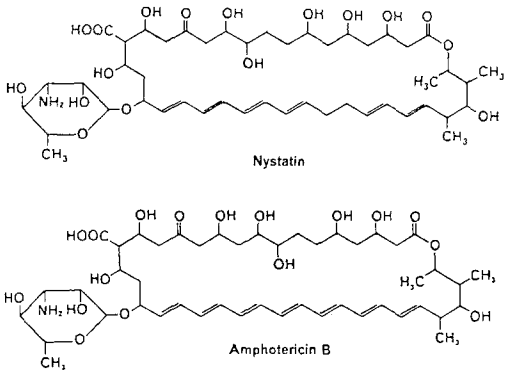

Fig. 1. Structural formulae of nystatin and amphotericin B (as given in Medoff & Kobayashi, 1980)

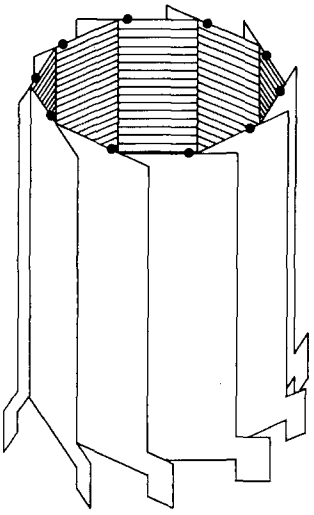

Fig. 2. Diagram of a single-length nystatin or amphotericin B channel. Each polyene molecule is schematized as a plane with a protuberance and a solid dot. The protuberance represents the amino sugar and the solid dot represents the lone hydroxyl group at the nonpolar end of the molecule. The shaded surface, partially visible in the center of the channel, represents the hydrophilic polyhydroxyl region. Note that the interior of the channel is polar whereas the exterior is completely nonpolar; the cleft between each pair of polyene molecules can accommodate a sterol molecule. The double-length channel is formed from two single-length channels hydrogen bonded in the middle of the membrane through the ring of hydroxyl groups (black dots). (From Finkelstein & Holz, 1973.)

and amphotericin B are ineffective from one side, because the channel is not long enough to span the bilayer. (3) If single-length and double-length channels have essentially the same structure, their inner diameters should be identical, and consequently their *relative* permeabilities to nonelectrolytes should be the same.

In this paper we describe experiments with nystatin on lipid bilayer membranes that address these

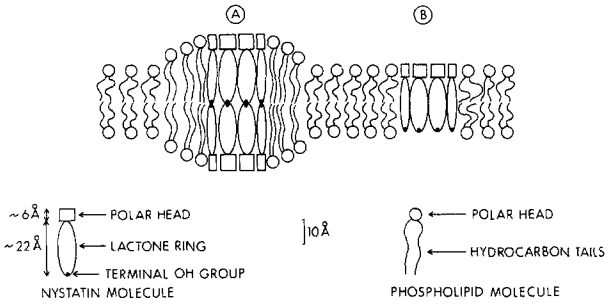

Fig. 3. Diagram of a double-length channel (*A*) and a single-length channel (*B*). A region of unmodified bilayer is depicted between *A* and *B*. Note that the acyl chains of the phospholipid can lengthen considerably beyond their value in the unmodified bilayer region to accommodate the long, double-length channel

three points, and we find that their results are consistent with the model proposed by Marty and Finkelstein (1975). Namely, nystatin (and amphotericin B) forms two types of channels, structurally very similar, that differ in length by a factor of two. We also report, for the first time, conductances of single-length channels.

Materials and Methods

I. Purification of Squibb Nystatin

Nystatin lot #21-329-45491-012, a kind gift from Dr. S. Lucania of the Squibb Institute for Medical Research (New Brunswick, N.J.), was stored as a dry powder under nitrogen in the dark at 4°C. Stock solutions in methanol (0.05 to 2 mg/ml) stored at −20°C retained activity for over a year. Previous work by Mechlinski and Schaffner (1974), using high performance liquid chromatography (HPLC), showed that Squibb nystatin is a mixture of 80% of the major component with two other tetraenes and one heptaene as minor components.

We made a preparative separation of Squibb nystatin by HPLC using a protocol modified after Mechlinski and Schaffner (1980). A stainless steel column, 60 cm long by 0.75 cm inner diameter, was handpacked with Waters Associates (Milford, Mass) reverse phase μBondapak C_{18}/Porasil B packing material, which consists of silica beads to which 18 carbon aliphatic chains are covalently bound. The column was equilibrated with 30% acetonitrile/70% 0.1 M Na_2HPO_4, pH 6.0. The two solvents, HPLC grade acetonitrile (Baker) and 0.1 M Na_2HPO_4 (Fisher), pH 6.0, were driven by two Waters Assoc. Model 6000A HPLC pumps whose relative flow rates were controlled by a solvent programmer. One ml of Squibb nystatin, 2 mg/ml in methanol, was loaded onto the column through a sample injector. The solvent programmer then linearly varied the solvent composition over a designated period (20 to 40 min) to a final composition of 50% acetonitrile/50% 0.1 M Na_2HPO_4, which was then run until the end of the experiment. In all experiments, the nystatin peaks continued to elute after the solvent mix had reached its final composition. Before the main separation, the column was pre-

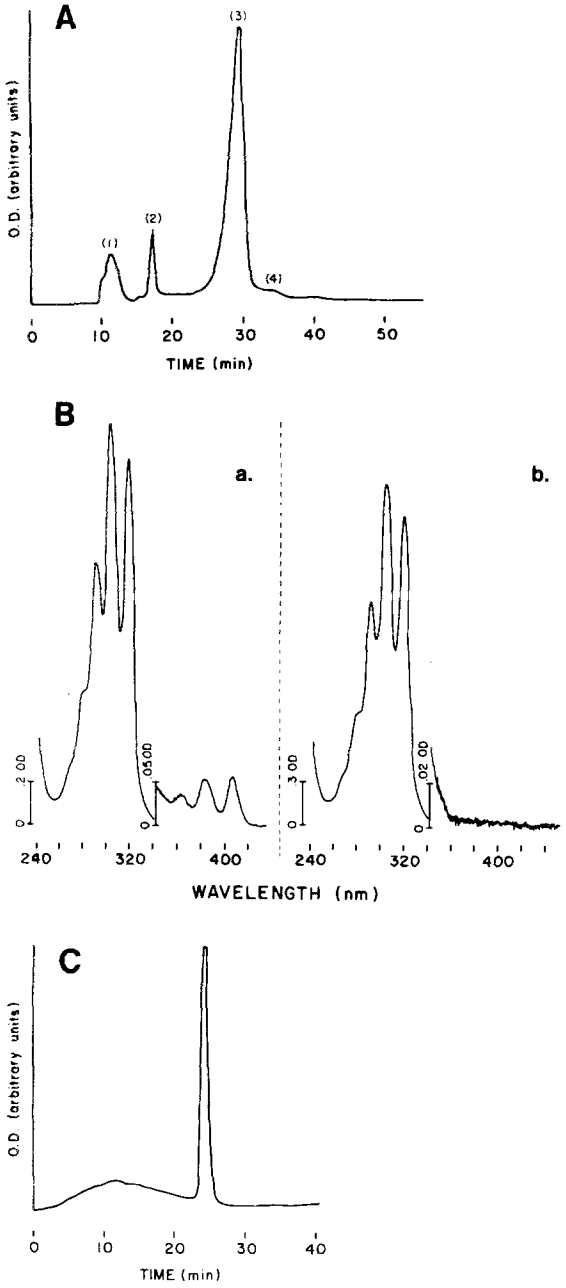

Fig. 4 (*left*). (*A*): Preparative separation of Squibb nystatin by HPLC. One ml of 2 mg/ml Squibb nystatin in methanol was injected onto a C_{18} μBondapak/Porasil B column. The column was eluted at a flow rate of 1.5 ml/min with a 30-min duration linear solvent gradient starting from an initial 30% acetonitrile/70% 0.1 M Na_2HPO_4, pH 6 solution and ending with a 50% acetonitrile/50% 0.1 M Na_2HPO_4, pH 6 solution. The final solvent mixture was run until the end of the separation. The eluant was collected as 1.5 ml volume fractions. The vertical scale is 2 OD (254 nm) full scale. The main fraction is designated by *3*. The two minor tetraene components are *1* and *2*. The minor heptaene component is designated by *4*. (*B*): Absorbance spectra of commercial Squibb nystatin (*a*) and HPLC-purified fraction #26 (*b*). The concentration of the Squibb nystatin sample is 0.022 mg/ml in methanol; the spectrum of the sample in *b* is from a 10-fold dilution of the HPLC-purified fraction #26. Methanol was used as the reference, and the samples were scanned at a rate of 30 nm/min. The visible-spectrum is shown at a more sensitive scale to illustrate the presence of the heptaene triplet (360–420 nm) in Squibb nystatin and its absence in fraction #26. (*C*): Analytical HPLC separation of HPLC-purified fraction #27.50 μl was injected onto the column and eluted with a 15-min linear solvent gradient. The initial and final solvent compositions are the same as those in *A*. The vertical scale is 0.05 OD (254 nm) full scale, which is 40 times more sensitive than that in *A*. Note that only one separation peak is seen; none of the minor fraction peaks seen in *A* appear here. (The broad hump preceding the nystatin peak is also seen when only methanol is injected.) An HPLC analysis of fraction #25 gave the identical result, and fraction #25, 26 and 27 were pooled together

conditioned with three separate injections of small amounts of Squibb nystatin, in order to improve the resolution of the peaks (W. Mechlinski, *personal communication*). The separated nystatin peaks were detected by a continuous flow UV spectrophotometer with 254-nm filters, and the output was recorded on a chart recorder. Fractions were collected with an LKB Model 7000 fraction collector (Rockville, Md.).

The preparative separation of Squibb nystatin is shown in Fig. 4*A*. Note the presence of three easily discernable peaks and a small bump to the right of the major fraction; this last represents the heptaene component, which shows poorly because it is present in small amounts and its extinction coefficient at 254 nm is less than that of the tetraenes.

The spectra of individual 1.5 ml fractions were taken on a Gilford Model 250 Spectrophotometer (Oberlin, Ohio). The main nystatin component was found in fractions 25, 26, and 27. One of the minor tetraene components appeared in fractions 8, 9, 10 and the other appeared in fraction 14; the heptaene component appeared primarily in fractions 31-36. Absorbance spectra for Squibb nystatin and HPLC nystatin fraction 26 are shown in Fig. 4*B*. The Squibb nystatin heptaene triplet, seen in the visible region, is absent in fraction 26; the presence or absence of the two minor tetraene components cannot readily be determined by comparing the two spectra, because all three tetraene spectra are so similar. Consequently, a 50-μl aliquot of pooled HPLC nystatin fractions 25-27 was injected onto the HPLC column to test its purity under the identical conditions used for the preparative separation (Fig. 4*C*). Only one separation peak was seen; i.e., HPLC nystatin fractions 25-27 contain a single tetraene entity (the major component of Squibb nystatin) on analytical HPLC separation. HPLC nystatin fractions 8–10 are predominantly composed of the first minor tetraene component, with significant amounts of the second minor tetraene component and small amounts of the major component; fraction 14 consists mostly of the second minor tetraene component with some contamination from the first.

From the peak OD at 318 nm, we estimated the concentration of HPLC nystatin fractions 25-27 to be about 0.2 mg/ml. Ten-to-one dilutions of this solution were made in methanol. The undiluted 0.2 mg/ml solution, stored under N_2 at 4°C, lost significant activity after eight months, while the diluted 0.02-mg/ml solutions, stored under N_2 at −20°C, lost some activity only after 15 months.

Table 1. List of monoglycerides used to make bilayers

Monoglyceride	Abbreviation	Chain	Fatty acid
Monomyristolein	GM-14	14 : 1	9-*cis*-tetradecenoic
Monopalmitolein	GM-16	16 : 1	9-*cis*-hexadecenoic
Monoolein	GM-18	18 : 1	9-*cis*-octadecenoic
Monoeicosenoin	GM-20	20 : 1	11-*cis*-eicosenoic
Monoerucin	GM-22	22 : 1	13-*cis*-docosenoic
Mononervonin	GM-24	24 : 1	15-*cis*-tetracosenoic

II. Measurements of Planar Bilayer Membranes

All experiments were done at room temperature.

A. Conductance Measurements

Membranes were formed by folding together two monolayers across a hole of 0.2 mm diameter in a 50 μM thick Teflon partition (Montal, 1974); the partition was pretreated with squalene (Reyes & Lattore, 1979) purified on an alumina column. The monolayers were spread from a 1% lipid solution in *n*-hexane, and approximately 15 min was allowed for hexane evaporation before a membrane was formed. Unless otherwise indicated, membranes separated symmetric salt solutions (~5 ml each) consisting of 2 M KCl, 3 mM $MgCl_2$, 10 mM HEPES, pH 7.0; both solutions could be independently stirred with magnetic stirring bars. After membrane formation, nystatin was added from stock solution to one or both sides of the membrane to final concentrations ranging from 0.01 to 40 μg/ml; the final concentration of methanol was never greater than 1%, and control experiments showed this to be without measurable effect on membrane conductance. Electrical measurements were made under voltage-clamp conditions through a single pair of electrodes. The electrodes were either saturated calomel or Ag/AgCl electrodes coupled to the solutions through 3 M KCl bridges, or Ag/AgCl electrodes directly contacting the solutions.

Most membranes were formed from a 4 : 1 molar mixture of lipid and ergosterol. This ratio was varied in some experiments over the range 8 : 1 to 2 : 1, and in a few experiments cholesterol replaced ergosterol. The lipids used were either (1) asolectin [soybean lecithin type IIS from Sigma (St. Louis, Mo.) from which neutral lipids were removed by acetone extraction (Kagawa & Racker, 1971)] or (2) all *cis*-monounsaturated monoglycerides of different chain lengths (GM-16 to GM-24; Table 1) purchased as ampulated solids from Nu-Chek-Prep (Elysian, Minn.). Acetone-extracted asolectin [40% phosphatidylcholine, 33% phosphatidylethanolamine, 14% phosphatidylinositol, and 4% cardiolipin (Miller & Racker, 1976)] was stored as a dry powder at −20°C; solutions in hexane stored at −20°C maintained their membrane-forming ability over a period of months. The GM-16, GM-18, and GM-22 ampulated stocks deteriorated at −20°C in about six months, while the GM-14, GM-20, and GM-24 were unchanged after one year. Solutions of all the monoglycerides in hexane stored at −20°C were usually stable for only two months.

Cholesterol was purchased from Sigma and ergosterol from Aldrich (Milwaukee, Wisc.). Both were recrystallized twice from ethanol and then stored as crystals in the dark at −20°C. Stock solutions of these sterols in chloroform were replaced approximately every three months.

B. Membrane Thickness Measurements

The thicknesses of the hydrophobic interior of monoglyceride/ergosterol bilayers were determined from membrane capacitance measurements. The membrane was stimulated with a symmetric voltage saw tooth, and capacitance was calculated from the magnitude of the jump in current that occurred when dV/dt changed sign. A set of known capacitors was used after every experiment to calibrate the electronics, and the calibration factor was used to correct the measured capacitance. The intrinsic capacitance of 16 pF for the membrane chamber and electrodes was subtracted from this corrected total to give the corrected membrane capacitance (C_m). The thickness (d) of the hydrophobic interior of the membrane was calculated from the parallel plate capacitor equation

$$C_m = \frac{\varepsilon \varepsilon_o A}{d}$$

where ε_o is the permittivity of free space (8.85×10^{-14} F/cm), A is the membrane area (3.7×10^{-4} cm^2) as measured by an eyepiece graticule on a Wild-Heerbrug M-5 microscope (Basel, Switzerland), and ε is the dielectric constant of the hydrophobic interior of the bilayer and was taken to have a value of 2, the dielectric constant of long-chain hydrocarbon (Fettiplace, Andrews & Haydon, 1971).

C. Nonelectrolyte Permeability Measurements

Nystatin-induced urea and glycerol permeabilities were simultaneously measured by a procedure slightly modified from that described by Holz and Finkelstein (1970). A 0.18-mm diameter asolectin/ergosterol or asolectin/cholesterol (molar ratios 2 : 1) membrane was formed between two compartments containing approximately 5 ml each of 2 M KCl, 3 mM $MgCl_2$, 10 mM HEPES, pH 7.0; both sides were continuously stirred throughout the experiment. Nystatin was added to one or both sides of the membrane from a 2-mg/ml stock methanolic solution. (For permeability measurements on unmodified membranes, a comparable amount of methanol, without nystatin, was added to one or both sides.) After the membrane conductance reached a steady state, ^{14}C-urea and ^{3}H-glycerol (Amershan, Arlington Heights, Ill.) were added from stock aqueous solutions to one side of the membrane to final concentrations of about 7 μCi/ml and 140 μCi/ml, respectively. About 1 min after the addition of these isotopes, two 20-μl samples were taken from that compartment for counting, and at 4-min intervals, 400-μl samples were taken from the opposite compartment for counting. (Each 400-μl sample was replaced with 400 μl of "cold" salt solution.) ^{14}C and ^{3}H were simultaneously counted on a Beckman LS7500 Microprocessor-controlled liquid scintillation counter with appropriate window settings. From these data the fluxes of ^{14}C-urea and ^{3}H-glycerol across the membrane were determined as described by Holz and Finkelstein (1970), and the nystatin-induced urea and glycerol permeabilities were calculated (*see* Results).

During an experiment, the voltage across the membrane was generally kept at zero; periodically, membrane conductance was determined by stepping the voltage to 10 mV and measuring the resulting current. Ag/AgCl pellet electrodes without Agar

bridges were used in all permeability experiments, because of their very low resistance. The access resistance (the resistance across the 0.18-mm diameter hole in the absence of a membrane) was typically 500 Ω in 2 M KCl and was measured at the end of every experiment. The measured membrane conductance was corrected for the access resistance; this correction ranged from 10 to 40%.

Results

Macroscopic Conductance Induced by Nystatin

Fraction 25-27 of HPLC-purified nystatin, which contains the main component of Squibb nystatin, shares all of the parent material's action. The results described below for "nystatin" pertain equally well to both.

A. One-Sided Effect

When nystatin is added to one side of an asolectin/ergosterol membrane to a concentration greater than 0.1 μM, the conductance rises exponentially, after a short delay (depending on mixing time and the amount of nystatin added), with a time constant τ of about 25 sec to a steady state (Fig. 5).[1] The steady-state conductance is proportional to about the 4th or 5th power of the nystatin concentration (Fig. 6); this value for the power is fairly reproducible, but the actual conductances achieved in a given experiment for a given aqueous concentration of nystatin can vary widely.

Membranes treated with nystatin from one side are nonideally cation selective (*see also* Marty & Finkelstein (1975)). For an uncharged membrane (e.g., GM-18/ergosterol) with 10 mM KCl on the dilute side, gradients of KCl activity yield potentials of 51 mV/decade (Fig. 7); gradients of NaCl and LiCl activity yield potentials of 44 and 40 mV/decade, respectively. Thus, the order of cation permeability is $P_{K^+} > P_{Na^+} > P_{Li^+}$. This sequence is confirmed by bi-ionic potential measurements: a membrane separating 100 mM KCl from 100 mM NaCl develops a bi-ionic potential of 8 mV (NaCl side positive), and one separating 100 mM KCl from 100 mM LiCl develops a potential of 26 mV (LiCl side positive). Although the one-sided action of nystatin renders the membrane much more permeable to *univalent* cations than to anions, such a membrane is virtually impermeable to *divalent* cations. For ex-

[1] There can be considerable variability in τ; in general, the kinetics of nystatin action are very complicated and are not considered in this paper.

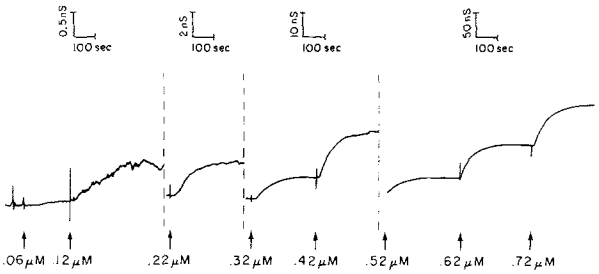

Fig. 5. A continuous record showing the increases in conductance after additions of small amounts of Squibb nystatin to one side of an asolectin/ergosterol (molar ratio 2 : 1) membrane. The 180-μM diameter membrane was clamped at +10 mV. The salt solution on both sides of the membrane was 2 M KCl, 0.01 M HEPES, 0.002 M $MgCl_2$, pH 7.0. Note that there are changes of the vertical scale occurring at the dashed lines. The aliquots of nystatin were added at the arrows, and concentrations listed beneath the arrows represent the total aqueous concentration of nystatin after the addition. A log conductance *vs.* log concentration plot gives a slope of 3.8

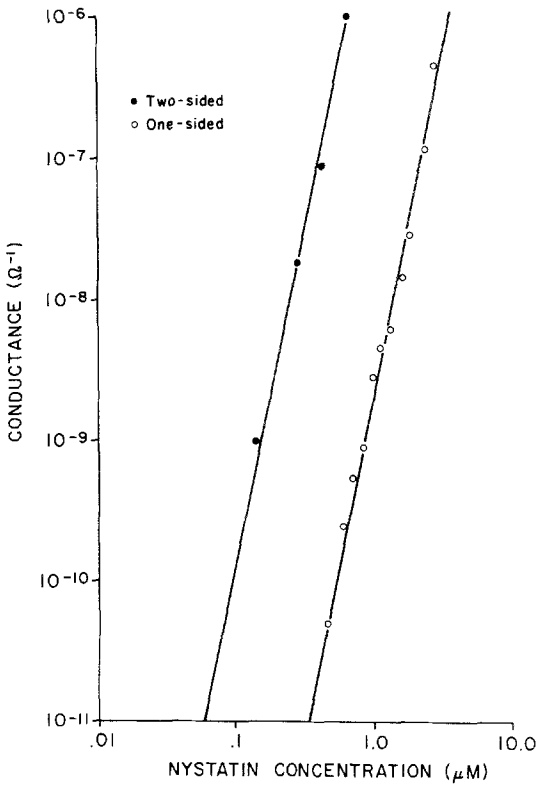

Fig. 6. Log conductance *vs.* log concentration plot for one- and two-sided additions of HPLC-Purified Nystatin to asolectin/ergosterol (molar ratio 2.5 : 1) membranes. The salt solution was 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7, and the membrane diameter was 190 μM. ○, one-sided data collected from a single experiment. ●, two-sided data from a single experiment. Both lines give a slope of 5. This graph illustrates the general observation that one obtains a higher two-sided- than one-sided-induced conductance for a given concentration of nystatin

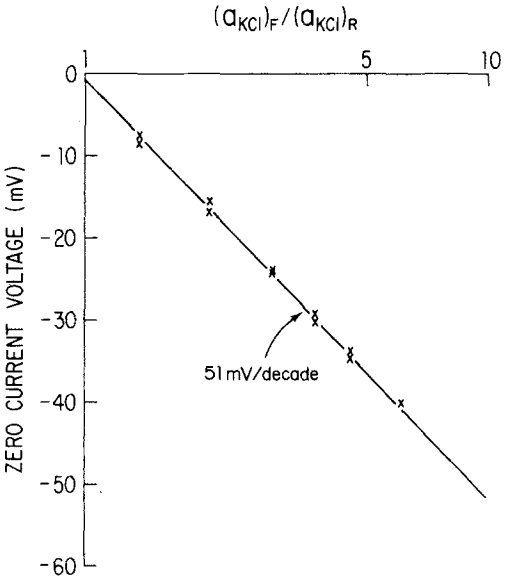

Fig. 7. One-sided, nystatin-induced cation selectivity. The results of two KCl selectivity experiments are shown. Squibb nystatin was added to the front aqueous compartment to concentrations of 4 and 12 μM. The membranes, GM-18/ergosterol (molar ratio 4 : 1), initially separated symmetric salt solutions of 10 mM KCl, 1 mM HEPES, 0.3 mM $MgCl_2$, pH 7.0. The KCl concentration in the front (F) compartment was raised by adding aliquots of 2 M KCl to that compartment. The graph is a semilogarithmic plot of membrane potential (zero-current voltage) *vs.* the ratio of KCl activities (a_{KCl}) in the front (F) and rear (R) compartments. Potentials are those of the front compartment relative to that of the rear

ample, in $MgCl_2$ gradients, Cl^--selective potentials of 52 mV/decade develop. The degree of ion selectivity is independent of the sterol (ergosterol or cholesterol) and, except at very low salt concentrations, it is also independent of membrane surface charge. Thus, GM-18/sterol (an uncharged membrane) and asolectin/sterol (a negatively charged membrane) show the same selectivity for K^+ over Cl^-.

B. Two-Sided Effect

The response of an ergosterol-containing membrane to two-sided addition of nystatin is similar to that obtained with one-sided addition, except that the kinetics are much slower ($\tau \approx 400$ sec), and there is often a slow linear creep of conductance that does not level off even after two hours. Subject to this disclaimer, steady-state conductance is proportional to about the 4th power of the nystatin concentration (Fig. 6), a value similar to that obtained with one-sided addition. Comparable conductances are generally achieved with much smaller concentrations of nystatin added to both sides than to one side (*see also* Marty & Finkelstein, 1975). This difference in sensitivity to nystatin's one-sided and two-sided action is much more pronounced in cholesterol-containing membranes than in ergosterol-containing membranes. In contrast to membranes treated from one side, membranes treated with nystatin from both sides are nonideally *anion* selective (*see also* Cass et al., 1970); gradients of KCl activity yield potentials of 50 mV/decade, and this degree of selectivity is independent of the sterol (ergosterol or cholesterol) and membrane surface charge. Although the two-sided action of nystatin renders the membrane much more permeable to *univalent* anions than to cations, such a membrane is virtually impermeable to *divalent* anions. Thus, in Na_2SO_4 gradients, virtually ideal Na^+ selective potentials develop (Cass et al., 1970).

C. Other HPLC Fractions

Fractions 8-10, 14, and 32-35, which contain the various polyene contaminants present in the parent material (*see* Methods), had neither one- or two-sided activity on asolectin/ergosterol (2.5 : 1) membranes. Thus, fraction 25-27, the main component of the parent material, is the only purified fraction displaying any one- and two-sided activity. It therefore appears that the unusual one-sided *vs.* two-sided behavior of Squibb nystatin can be attributed to one molecule (the main component), and not in part to one or more of the polyene contaminants in the original preparation.

Parenthetically, if a large amount of Squibb nystatin is added to one or both sides of a sterol-free asolectin membrane separating 2 M KCl solutions, a small conductance develops. It is produced by channels having a mean conductance of 7.5 pS and a mean lifetime of 35 sec (on a survivor plot); the occurrence of these channels is totally independent of whether the membrane contains any sterol. This channel activity was not found in fraction 25-27 or in any of the fractions described in the previous paragraph, suggesting that it either is a polyene present in only undetectable amounts, became permanently stuck on the HPLC column during purification, or is a nonpolyene contaminant in one of the untested fractions.

II. Single Channel Data From HPLC (Fraction 25-27) Purified Nystatin

A. One-Sided Addition

Figure 8*A* shows a record of single channels induced in an asolectin/ergosterol (molar ratio 4 : 1)

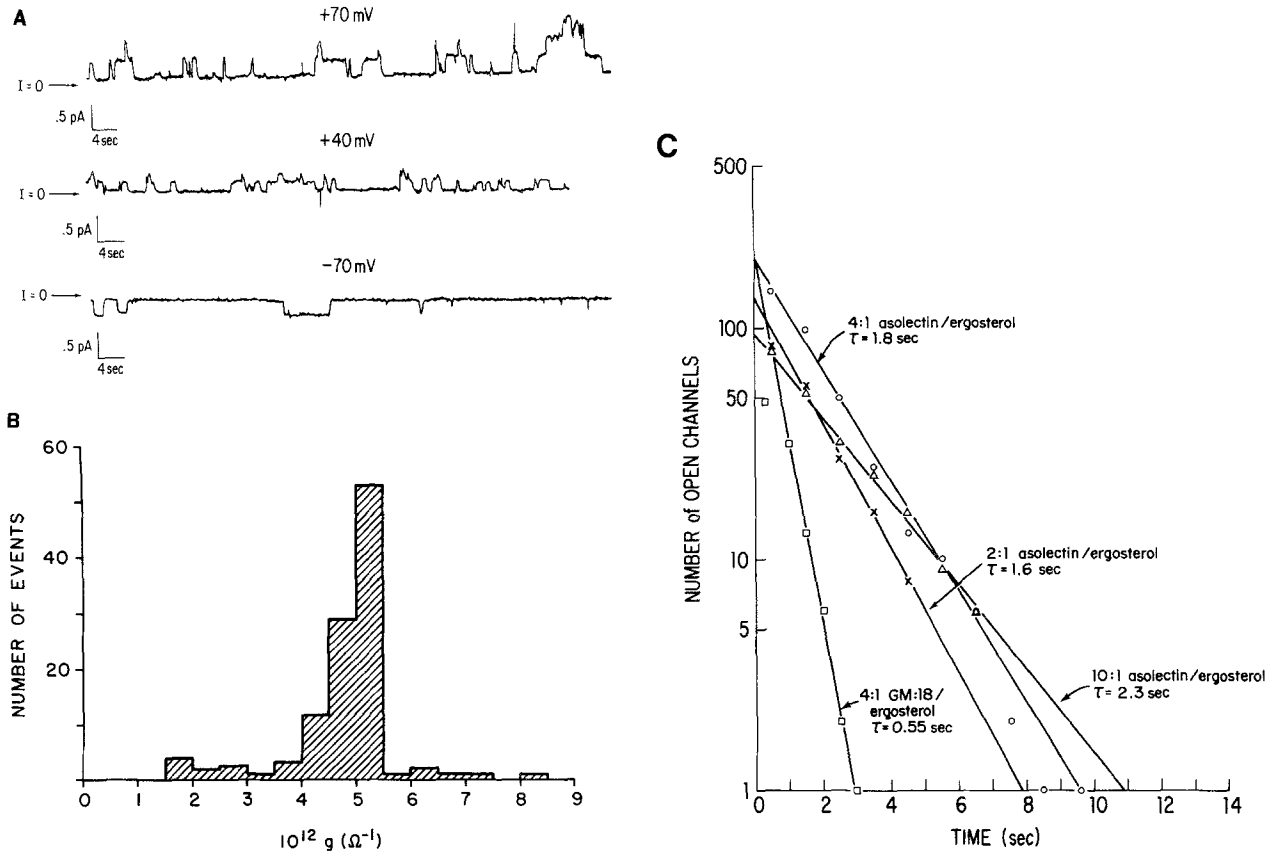

Fig. 8. (*A*): Current records of one-sided, nystatin-induced single channels at three different voltages. The three records were taken from the same asolectin/ergosterol (molar ratio 4 : 1) membrane separating symmetric salt solutions of 2 M KCl, 0.01 M HEPES, 0.003 M $MGCl_2$, pH 7.0. The HPLC-purified nystatin concentration on one side was 0.2 μM, and the voltages are those of that side relative to that of the opposite side. (The greater number of channels seen at +70 than at −70 mV is consistent with the weak voltage dependence observed in macroscopic conductance records; it is not considered further in this paper.) (*B*): Histogram of the number of one-sided, nystatin-induced single channels *vs.* their respective conductances. 0.2 μM HPLC-purified nystatin was one one side of an asolectin/ergosterol (molar ratio 4 : 1) membrane separating symmetric salt solutions of 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7. The frequency response was limited to 10 Hz. The current resided at bare membrane level most of the time, but occasionally a single channel would open for a short duration and then close. Only rarely were two channels open at the same time. The histogram was constructed from a total of 113 single channels. The mode conductance is about 5 pS. (*C*): Survivor plot of lifetimes of one-sided, nystatin-induced single channels on 10 : 1, 4 : 1, and 2 : 1 asolectin/ergosterol and 4 : 1 GM-18/ergosterol membranes. Each line is the result of one experiment. For all experiments, the salt solution was 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7. The one-sided HPLC-purified nystatin concentrations for the 10 : 1, 4 : 1, and 2 : 1 asolectin/ergosterol membranes were approximately 0.15–0.2 μM; the one-sided concentration for the 4 : 1 GM-18/ergosterol membrane was 0.8 μM. All measurements were made with the voltage of the nystatin-containing compartment clamped at 50 mV

membrane by the one-sided action of HPLC-purified nystatin; in 2 M KCl most of the channels have a conductance of about 5 pS (Fig. 8*B*), and their *I–V* characteristic is linear over the range ±80 mV. Channel lifetimes (Fig. 8*C*) in asolectin/ergosterol membranes vary only slightly with ergosterol concentration (τ = 1.6, 1.8, and 2.3 sec for asolectin/ergosterol molar ratios of 2 : 1, 4 : 1, and 10 : 1, respectively) but are considerably shorter (τ = 0.55 sec) in GM-18/ergosterol membranes (molar ratio 4 : 1). In this latter membrane, the single channel conductance in 2 M KCl is 4.5 pS, slightly smaller than that in asolectin/ergosterol membranes. We saw no significant rapid opening and closing (flickering) of asolectin-ergosterol channels at higher time resolution (filtering at 100 Hz). The cation selectivity of these channels, 8 mV for a KCl gradient of 2 *vs.* 0.5 M, agrees with the macroscopic selectivity, thus indicating that the macroscopic conductance arises from an ensemble of these channels.[2]

[2] This 8 mV, out of a possible 32 mV, is less than the one-sided selectivity measured at lower KCl concentrations, because the cation selectivity of the one-sided-induced nystatin conductance is less at high salt concentrations (*see* section V-*B*).

B. Two-Sided Addition

The single channel conductance of channels formed by the two-sided action of nystatin is 1 pS in 2 M KCl (Fig. 9); the same value is obtained both on asolectin/cholesterol membranes (molar ratio 2 : 1) and on GM-20/ergosterol membranes (molar ratio 4 : 1). This conductance value for channels formed by nystatin's two-sided action is about one-fifth that for channels formed by its one-sided action (*see* previous section) and is about one-half the value reported by Kasumov et al. (1979) for its two-sided action on hydrocarbon-containing black lipid membranes. The channel lifetime of >100 sec is considerably longer than the ~2-sec lifetime of channels formed by one-sided action.

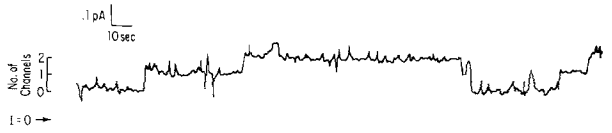

Fig. 9. Current record of two-sided, nystatin-induced single channels. The salt solution on both sides of an asolectin/cholesterol (molar ratio 2 : 1) membrane was 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7; the HPLC-purified nystatin concentration on both sides was 0.8 μM. The voltage across the membrane was clamped at 100 mV

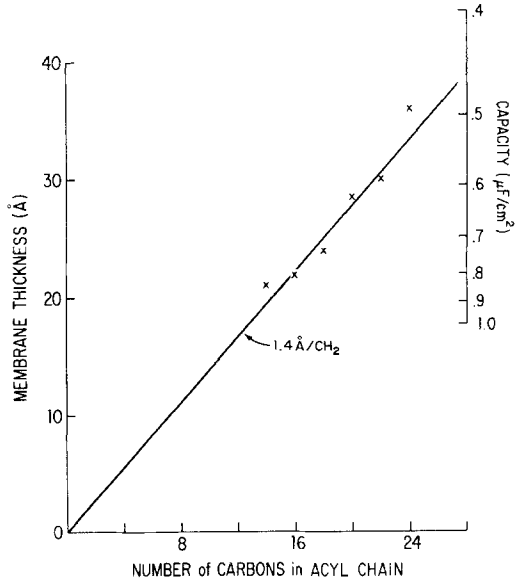

Fig. 10. Plot of the thickness of the hydrophobic interior of monoglyceride/ergosterol (molar ratio 4 : 1) membranes *vs.* the number of carbon atoms in the fatty acid acyl chain. All membranes separated symmetric salt solutions consisting of 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7. A capacitance scale is included on the right side of the figure. The data are from Table 2

III. Effect of Membrane Thickness on One- and Two-Sided Action of Nystatin

The thicknesses of the hydrophobic interior of membranes formed from GM-14/ergosterol to GM-24/ergosterol (molar ratio 4 : 1) increase monotonically with chain length (Table 2 and Fig. 10; *see also* Benz, Fröhlich, Läuger & Montal, 1975). Each membrane was tested for one- and two-sided nystatin action by first adding nystatin to one side and recording the resulting conductance, and then adding about 20% as much to the other side and recording the resulting much larger conductance. The results are summarized in Table 3, which lists the individual monoglyceride/sterol membranes and whether or not they are sensitive to the one- and two-sided action of nystatin.

All monoglyceride/sterol membranes tested are sensitive to the two-sided action of nystatin; conductances of at least 3×10^{-3} S/cm^2 are obtained with 2-3 μM nystatin on one side and 0.5–0.75 μM on the other. The conductance variability, discussed earlier, precludes our making a hierarchy of sensitivity for membranes ranging from GM-14/ergosterol to GM-24/ergosterol.

Table 2. Capacitance and thickness of the hydrophobic interior of monoglyceride/ergosterol membranes

	Capacitance (μF/cm^2)	Thickness (Å)
4 : 1 GM-14/ergosterol (5)	0.85 ± 0.04	21 ± 1
4 : 1 GM-16/ergosterol (6)	0.82 ± 0.02	22 ± 1
4 : 1 GM-18/ergosterol (2)	0.74 ± 0.02	24 ± 0.5
4 : 1 GM-20/ergosterol (10)	0.62 ± 0.03	28.5 ± 2
4 : 1 GM-22/ergosterol (4)	0.59 ± 0.00	30 ± 0
4 : 1 GM-24/ergosterol (6)	0.49 ± 0.02	36 ± 1

Numbers in parentheses refer to the number of experimental measurements. Capacitance values represent the mean, plus or minus the total range measured.

Table 3. The efficacy of nystatin's one- and two-sided action on lipid/sterol membranes

	One-sided	Two-sided
4 : 1 GM-14/ergosterol	+	+
4 : 1 GM-16/ergosterol	+	+
4 : 1 GM-16/cholesterol	+	+
4 : 1 GM-18/ergosterol	+	+
4 : 1 GM-18/cholesterol	−	+
4 : 1 GM-20/ergosterol	−	+
4 : 1 GM-22/ergosterol	−	+
4 : 1 GM-24/ergosterol	−	+
4 : 1 asolectin/ergosterol	+	+
4 : 1 asolectin/cholesterol	+/−	+

In contrast to the two-sided action of nystatin, the one-sided action displays a sharp chain-length dependence. Whereas GM-14/, GM-16/, and GM-18/ergosterol membranes are very sensitive to the one-sided action of nystatin (e.g., conductances of 10^{-3} S/cm^2 for 2–3 μM nystatin on one side), GM-20/, GM-22/, and GM-24/ergosterol membranes display *no* one-sided-induced conductance at 2 μM nystatin. Only when huge amounts of nystatin are added to one side of these membranes is any conductance seen at all. For instance, 20 μM HPLC-purified nystatin added to one side of a GM-20/ergosterol membrane induced a conductance of 3×10^{-5} S/cm^2, which is trivial compared to that induced on GM-14/, GM-16/, or GM-18/ergosterol membranes by much smaller concentrations. Comparably low conductances were obtained with Squibb nystatin on GM-20/, GM-22/, and GM-24/ergosterol membranes. These one-sided-induced conductances on longer chain bilayers probably result from membrane disruption by the high concentration of surface active material, and not from the same one-sided action seen on shorter chain bilayers. This is suggested by our observation that further additions of nystatin did not cause the conductance to increase with the 4th power dependence seen on shorter chain bilayers.

GM/cholesterol membranes give similar results to those obtained on GM/ergosterol membranes, except that only GM-14/ and GM-16/cholesterol membranes are sensitive to the one-sided action of nystatin, whereas GM-18/cholesterol membranes are insensitive, as are the higher chain derivatives. GM-14/ and GM-16/cholesterol membranes are less sensitive than corresponding GM/ergosterol membranes, in keeping with the general lower sensitivity to nystatin action of cholesterol-containing membranes.

IV. Permeability of Nystatin-Treated Membranes to Nonelectrolytes (Urea and Glycerol)

From the simultaneous measurements of the fluxes (Φ^*_{urea} and Φ^*_{glycerol}) of ^{14}C-urea and ^{3}H-glycerol, as described under Results, and the measured concentrations (c^*_{urea} and c^*_{glycerol}) of the isotopes in the "hot" compartment, the products of permeability coefficients (P_d(urea) or P_d(glycerol)) and membrane area (A) were determined from the equation

$$P_dA = \frac{\Phi^*}{c^*}.$$

One-sided experiments were performed on asolectin/ergosterol (molar ratio 2 : 1) membranes, whereas two-sided experiments were performed on asolectin/cholesterol (molar ratio 2 : 1) membranes. Cholesterol was used as the sterol in the two-sided experiments, because no one-sided-induced conductance was manifested on these membranes at the nystatin concentrations employed, and hence we were assured that the fluxes were not contaminated by contributions from one-sided-induced channels. Unstirred layer corrections, which were maximally 33%, were made as described by Holz and Finkelstein (1970).

Figures 11*A* and *B* plot P_dA *versus* membrane conductance (G) for one- and two-sided nystatin-treated membranes, respectively; as expected, P_dA is a linear function of G. From the slopes of the lines we obtain

$$\left[\frac{(P_dA)_{\text{urea}}}{(P_dA)_{\text{glycerol}}}\right]_{\text{one-sided}} = 8.3$$

$$\left[\frac{(P_dA)_{\text{urea}}}{(P_dA)_{\text{glycerol}}}\right]_{\text{two-sided}} = 8.0.$$

Thus, one-sided-induced channels display the same relative permeability to urea and glycerol as do two-sided-induced channels, indicating that the channel radii are the same. Our value of $(P_dA)_{\text{urea}}/(P_dA)_{\text{glycerol}}$ for the two-sided effect of nystatin on hydrocarbon-free membranes is identical to that obtained by Holz and Finkelstein (1970) on decane-containing membranes.

V. Additional Observations

A. Amphotericin B

The results of our few amphotericin B experiments were in essential agreement with corresponding nystatin experiments. Like nystatin, amphotericin B induced a one-sided, cation-selective conductance (53 mV/decade KCl) and a two-sided, anion-selective conductance (47 mV/decade KCl); also like nystatin, it was active from one side on GM-18/ergosterol membranes, but not on GM-20/ergosterol membranes. (Amphotericin B even produced, like nystatin, a contaminant channel when added to sterol-free asolectin membranes; its conductance was 24 pS in 2 M KCl.) Previous work had shown that amphotericin B and nystatin two-sided action are very similar (Cass et al., 1970; Holz & Finkelstein, 1970); the present results indicate the same for their one-sided action.

B. Effect of Salt Concentration on Ion Selectivity

The cation selectivity of the one-sided, nystatin-induced channel in KCl gradients decreases with in-

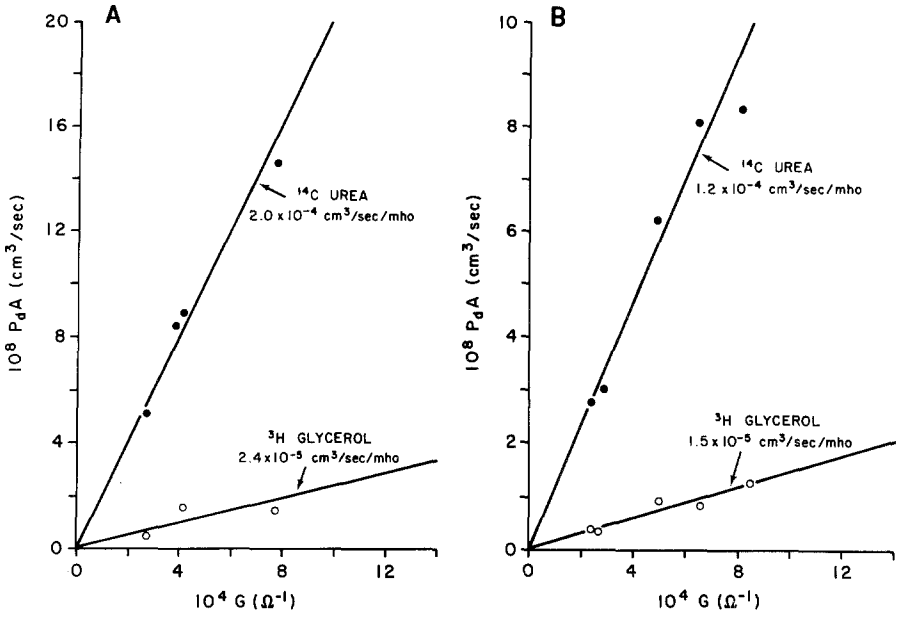

Fig. 11. (*A*): P_dA for urea and glycerol as a function of the conductance induced by the *one-sided* action of nystatin. Asolectin/ergosterol (molar ratio 2 : 1) membranes separated symmetric solutions of 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7. The measured values of P_dA for urea and glycerol were corrected for unstirred layers and for background bilayer permeabilities. The ratio of the slopes of the urea and glycerol lines is 8.3. (*B*): P_dA for urea and glycerol as a function of the conductance induced by the *two-sided* action of nystatin. Asolectin/cholesterol (molar ratio 2 : 1) membranes separated symmetric solutions of 2 M KCl, 0.01 M HEPES, 0.003 M $MgCl_2$, pH 7. The measured values of P_dA for urea and glycerol were corrected for unstirred layers and for background bilayer permeabilities. The ratio of the slopes for urea and glycerol is 8.0

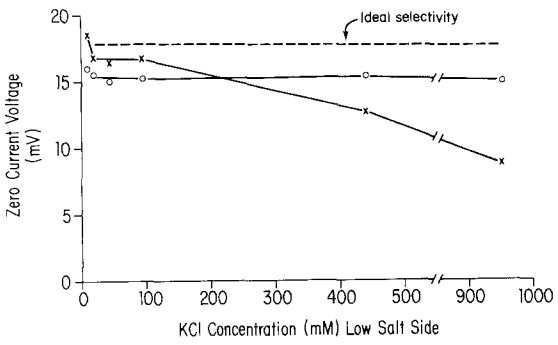

Fig. 12. Demonstration that the one-sided, nystatin-induced cation selectivity decreases with salt concentration, but the two-sided, nystatin-induced anion selectivity does not. ×, one-sided; ○, two-sided. The ionic selectivities are reflected in the zero current voltage produced in a 2 : 1 KCl activity gradient. The absolute KCl concentrations on the two sides of the membrane were varied, though the ratio of their activities always equaled 2. The value of the KCl concentration on the low salt side of the gradient is plotted on the abscissa. The absolute value of the zero current voltage is plotted on the ordinate, and the absolute value expected for ideal selectivity is shown by the dashed line at the top of the figure. Each point is the average of two experiments on asolectin/ergosterol (molar ratio 4 : 1) membranes. All solutions were buffered with 1 mM HEPES, 0.3 mM $MgCl_2$, pH 7. For the two highest KCl concentration points, the low salt side was supplemented with glucose to balance the osmolalities. Both the one- and two-sided-induced conductances showed an artifactual loss of selectivity in the absence of the osmotic balancing

creasing salt concentration, whereas the anion selectivity of the two-sided-induced channel does not (Fig. 12). This latter finding contrasts with the results of Finkelstein and Holz (1973) who observed a decrease in the anion selectivity of two-sided, nystatin-induced conductance with increasing concentrations of KCl. Their results, however, may have been complicated by salt polarization resulting from osmotic water flow, as they did not balance the osmolalities of the salt solutions with nonelectrolyte as in Fig. 12.

Discussion

I. Tests of the Single-Length and Double-Length Channel Explanation for the One- and Two-Sided Actions of Nystatin and Amphotericin B

The primary purpose of the experiments described in this paper was to test the validity of the model proposed by Marty and Finkelstein (1975) for the one- and two-sided actions of nystatin and amphotericin B on sterol-containing bilayer membranes. The crux of that model, summarized in Fig. 3, is that both actions are attributable to channels having

essentially the same structure. The channels differ primarily in length—the two-sided-induced channel being twice the length of the one-sided-induced channel. Because of flexibility in bilayer thickness, the same membrane can host both structures. Three predictions of this model were tested and all three were completely satisfied.

The first prediction, although trivial, is essential for any further considerations of the model: namely, the same molecule is responsible for both the one- and two-sided action; i.e., a contaminant of Squibb nystatin does not produce one of the actions. Using HPLC chromatography, we separated the two tetraene and one heptaene contaminants from the main fraction and showed that both the one-sided and two-sided activity reside entirely in the main fraction. These experiments provide convincing evidence that the same molecule (nystatin) is responsible for both effects.

The second prediction relates to the postulated difference in length of the channels responsible for the one- and two-sided actions of nystatin. If this model is correct, channel formation should be critically dependent on bilayer thickness, and this is precisely what we found for nystatin's one-sided action. Whereas GM-14/, GM-16/, and GM-18/ergosterol membranes were very sensitive to nystatin's one-sided action, GM-20/, GM-22/, and GM-24/ergosterol membranes were totally refractory. [Phosphatidylcholine-22/cholesterol liposomes are also insensitive to the one-sided action of amphotericin B (van Hoogevest & deKruijff, 1978)]. Moreover, the insensitivity of these thicker membranes cannot be attributed to failure of nystatin binding (*see also* van Hoogevest & deKruijff, 1978), since even after the compartment was washed free of nystatin by perfusion, addition of nystatin to the opposite compartment produced a large two-sided effect, thus demonstrating that nystatin had associated with the side of the membrane to which it was originally added. We believe that membranes formed by GM-20/ergosterol and longer chain derivatives are too thick for the one-sided, nystatin-induced channel to span. A comparison of the length of the nystatin portion presumed embedded in the hydrophobic interior of the bilayer with the thickness of that interior (as determined from capacitance measurements) fits nicely with this interpretation (Fig. 13).

It may appear surprising from Fig. 13 that the double-length channel can be accommodated by all, or any, of the sterol-containing monoglycerides ranging from GM-14 to GM-24. The thicknesses given in the figure, however, are those of the unperturbed bilayer; in that state the acyl chains of the lipid are compressed to about 60% of their fully

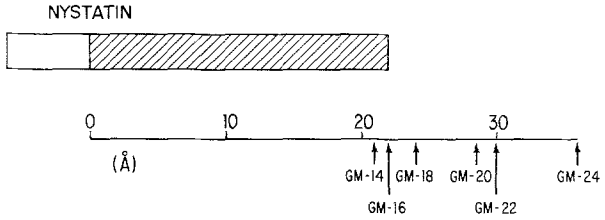

Fig. 13. Schematic comparison of the length of the hydrophobic portion (hatched) of nystatin, as measured on a CPK molecular model, with the experimentally measured thickness of the hydrophobic interior of monoglyceride/ergosterol membranes. The thicknesses of monoglyceride/ergosterol membranes are taken from Table 2

extended length. Given a local perturbation in the membrane by a structure such as a double-length channel, the surrounding acyl chains can apparently fully extend to accommodate it.

The third prediction of the model is that the inner diameters of the two channels are essentially identical, since one is the tail-to-tail dimer of the other. If nonelectrolyte selectivity by the channels is based primarily on steric factors, the same nonelectrolyte selectivity should be manifested by the two channel forms. A sensitive test of this prediction is the relative permeabilities to urea and glycerol. The channel formed by the two-sided action of nystatin markedly discriminates between these molecules of similar size (Holz & Finkelstein, 1970), indicating that their interaction with the channel is substantial; therefore, small differences in channel diameter should result in large differences in relative permeabilities. Our finding that the value of P_d(urea)/P_d(glycerol) is essentially the same, namely 8, for both the one-sided-induced and two-sided-induced channels is strong evidence that their internal diameters are the same.

In summary, the results of the three tests that we performed are completely in accord with the model that the one- and two-sided actions of nystatin on lipid bilayer membranes are produced by channels of essentially similar structure, and that the major difference between them is that the channel formed by the two-sided action of nystatin is twice the length of that formed by its one-sided action. (Why the single-length channel is cation selective whereas the double-length channel is anion selective remains obscure.) The few experiments with amphotericin B described in this paper, previous data on its action on bilayer membranes (Holz & Finkelstein, 1970), and its known chemical similarity to nystatin leave little doubt that amphotericin B also forms single-length and double-length channels very similar to those formed by nystatin.

Table 4. Permeability coefficients (p_d) of urea and glycerol for single-length and double-length nystatin channels

	p_d urea (cm^3/sec/channel)	p_d glycerol (cm^3/sec/channel)
2 M KCl		
Single-length channel	1.0×10^{-15}	1.2×10^{-16}
Double-length channel	1.2×10^{-16}	1.5×10^{-17}
0.1 M KCl		
Single-length channel	2.4×10^{-15}	—
Double-length channel	3.6×10^{-16}	—

II. Single-Channel Permeabilities to Urea and Glycerol

From single-channel conductance measurements plus urea and glycerol permeabilities measured at known macroscopic conductances, we can calculate[3] single-channel permeabilities to urea and glycerol (Table 4). From the model one would expect, because of the difference in channel lengths, that $(p_d)_{\text{single-length}}/(p_c)_{\text{double-length}} = 2$, whereas we find it to be 8.3 in 2 M KCl and 6.7 in 0.1 M KCl. Two possible explanations for this deviation from the theoretical prediction are (1) differential blockage of single-length and double-length channels by salt (this is suggested by the "better" result in 0.1 than in 2 M KCl) and (2) underestimation of the number of single-length channels relative to the number of double-length channels in the experiments. This could result if the conductance per channel in membranes containing large numbers of channels (the condition under which the tracer flux experiments were performed) is not equal to the measured single-channel conductance. At high surface densities, nystatin channels may cluster and thereby modify the value of each other's conductance from that of the isolated channel.

III. Comparison of Nystatin Action on Artificial and Biological Membranes

The macroscopic data for the one-sided action of nystatin on ergosterol-containing planar bilayer membranes agree with results of similar experiments on cell membranes. Permeability changes induced in fungi, red blood cells, and *A. laidlawii* by nystatin (Lampen, 1966; Cass & Dalmark, 1973; deKruijff et al., 1974) are consistent with the formation of single-length pores of radii ~4 Å in the plasma membrane. Cell death results primarily from the profound leakage of intracellular K^+ and the subsequent disruptions in cellular metabolism caused by the resulting ionic imbalance (Liras & Lampen, 1974). Also, in agreement with the data from biological membranes, ergosterol is more effective than cholesterol in potentiating nystatin's one-sided action on planar bilayers. Thus, the pore theory of one-sided nystatin and amphotericin B activity accounts for all of the pharmacological effects of these polyenes on cell membranes.

The major competing theory, as reviewed by Medoff and Kobayaski (1980), argues that nystatin and amphotericin B bind up and sequester membrane sterol, and that the remaining sterol-free membrane has the higher nonelectrolyte and ion permeability characteristics of nystatin- and amphotericin B-treated membranes. We do not believe that this is a tenable explanation. Although membrane permeability is a function of sterol content, sterol-free bilayers do not show the molecular sieving or the levels of nonelectrolyte and ion permeability seen in nystatin-treated membranes. Furthermore, we feel that the efflux of large intracellular molecules from cells treated with high concentrations of these polyenes (*see* Medoff & Kobayaski, 1980) is probably secondary to osmotic lysis, cell autolysis, and the detergent-like activity of the large surface concentrations of nystatin or amphotericin B. In sum, we believe that the artificial membrane data are extremely relevant to polyene pharmacology and that these data have spawned a pore theory of nystatin and amphotericin B activity that is consistent with the large body of data documenting the cytotoxic effects of these polyenes on cell membranes.

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[3] The double-length single-channel conductance in 0.1 M KCl, which was used to calculate p_d(urea) and p_d(glycerol) in that medium, could not be measured directly, because of its small size. It was estimated from the change in macroscopic conductance that resulted when the salt concentration was suddenly increased from 0.1 to 2.0 M KCl (and using the measured single-channel conductance in 2 M KCl), under the assumption that the number of channels in the membrane did not change immediately when the KCl concentration was increased.

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