

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

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ABSTRACT Nystatin and amphotericin B increase the permeability of thin (<100 Å) lipid membranes to ions, water, and nonelectrolytes. Water and nonelectrolyte permeability increase linearly with membrane conductance (i.e., ion permeability). In the unmodified membrane, the osmotic permeability coefficient, P_f , is equal to the tagged water permeability coefficient, $(P_d)_w$; in the nystatin- or amphotericin B-treated membrane, $P_f/(P_d)_w \approx 3$. The unmodified membrane is virtually impermeable to small hydrophilic solutes, such as urea, ethylene glycol, and glycerol; the nystatin- or amphotericin B-treated membrane displays a graded permeability to these solutes on the basis of size. This graded permeability is manifest both in the tracer permeabilities, P_d , and in the reflection coefficients, σ (Table I). The "cutoff" in permeability occurs with molecules about the size of glucose (Stokes-Einstein radius $\cong 4$ Å). We conclude that nystatin and amphotericin B create aqueous pores in thin lipid membranes; the effective radius of these pores is approximately 4 Å. There is a marked similarity between the permeability of a nystatin- or amphotericin B-treated membrane to water and small hydrophilic solutes and the permeability of the human red cell membrane to these same molecules.

The preceding paper (Cass et al., 1970) dealt with the ion permeability of thin lipid membranes treated with polyene antibiotics; this paper focuses on the water and nonelectrolyte permeability of membranes treated with nystatin or amphotericin B.

I. MATERIALS AND METHODS

All membranes were formed by the brush technique of Mueller et al. (1963) from a solution that contained 30 mg of lipid, 200 mg *dl*- α -tocopherol, and 14 mg additional cholesterol in 1 ml of 2:1 chloroform methanol; a single sample of ox-brain lipid (Mueller et al., 1963) was used in all experiments.

A. Tracer Experiments

Membranes were formed at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ across a hole ($\sim 1 \text{ mm}^2$) in a polyethylene partition 125μ thick that separated two Lucite chambers (Fig. 1). Magnetic fleas stirred both sides continuously during the course of an experiment. Throughout an experiment, membrane resistance was monitored by passing a step of current (ΔI) across the membrane through a pair of Ag/AgCl electrodes and recording the resulting steady-state membrane potential (ΔV) through the same electrodes. The resistance was then given by $\Delta V/\Delta I$. When significant, the resistance without a membrane ($\sim 2 \times 10^8 \Omega$) was subtracted from the measured resistance.

Membranes were formed in a solution 100 mM in NaCl, 5 mM in sodium phosphate (pH 7), and, generally, 10 mM in the solute whose membrane permeability was to be measured. A typical experiment proceeded as follows: after the membrane had become completely black, sufficient antibiotic (dissolved in methanol) was added to

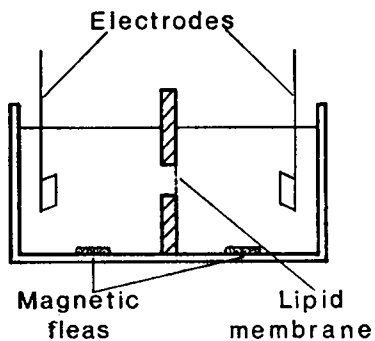


FIGURE 1. Schematic drawing of chamber used for tracer experiments. Note the aqueous channel behind the membrane.

both sides to reduce the resistance to the desired level. (Alternatively, the membrane was formed in the presence of the antibiotic.) When the resistance had attained a constant value, $10\text{--}30 \mu\text{Ci}$ of the appropriate tracer in a concentrated aqueous or methanolic solution was added at a time defined as 0 min to the 4.5 ml volume of the rear chamber. After approximately 30 sec of mixing, two $10 \mu\text{l}$ samples were taken from this chamber and placed in 15 ml of counting fluid. The top of the rear chamber was then sealed by a small piece of parafilm to prevent distillation of tracer into the front chamber. Every 4–5 min $50 \mu\text{l}$ samples were taken from the 3.0 ml volume of the front chamber for counting and replaced by $50 \mu\text{l}$ of “cold” buffer solution to avoid bulging the membrane. All data were corrected for these dilutions of the radioactivity in the front chamber. Experiments lasted from 20 min to 2 hr and ended when the membrane broke or the investigator wearied. Data from a typical experiment are shown in Fig. 2. The samples were counted in a Tricarb scintillation counter in a cocktail of 100 ml Spectrofluor (PPO and POPOP), 1800 ml toluene, and 600 ml absolute ethanol.

The isotopes, obtained from New England Nuclear Corp., were urea- C^{14} (5 mCi/millimole), *n*-butanol-1- C^{14} (3.7 mCi/millimole), ethylene-1,2- C^{14} glycol (2 mCi/millimole), thiourea- C^{14} (2.7 mCi/millimole), glycerol-2- H^3 (500 mCi/millimole), and THO (5 mCi/g). The nystatin was the commercial Squibb Mycostatin and the

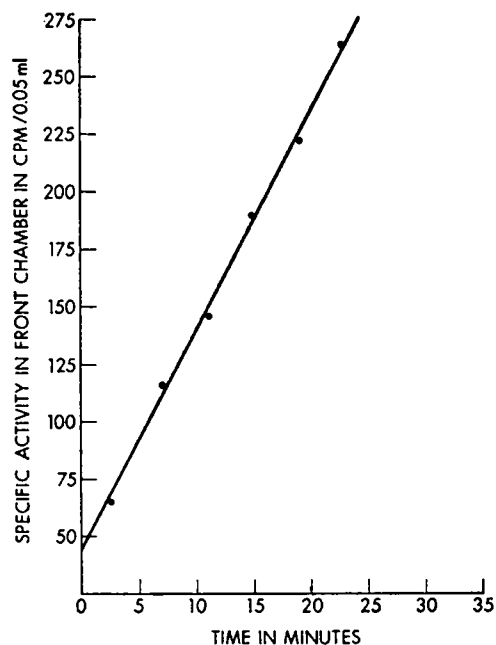


FIGURE 2. Results of urea- C^{14} diffusion experiment for a single nystatin-treated membrane. The membrane was formed in 100 mM NaCl, 5 mM sodium phosphate (pH 7). Nystatin was added to both chambers to a concentration of $3.0 \mu\text{g/ml}$, and after 20 min the membrane reached a steady resistance of $4.6 \times 10^8 \Omega$ ($55 \Omega\text{cm}^2$). At 0 time $10 \mu\text{Ci}$ of urea- C^{14} was added to the rear chamber and $50 \mu\text{l}$ samples were then periodically taken from the front chamber. Specific activity of rear chamber = $22 \times 10^4 \text{cpm}/0.05 \text{ml}$; volume of front chamber = 3.0 ml; area of membrane = 1.21mm^2 . From these data and slope of the line, $(P_d)_{\text{urea}}$ is found from equation (1) to be $1.75 \times 10^{-4} \text{cm/sec}$. (Normalizing to a resistance of $100 \Omega\text{cm}^2$ this becomes $0.96 \times 10^{-4} \text{cm/sec}$.) Temperature = $25^\circ\text{C} \pm 0.5^\circ\text{C}$.

amphotericin B was a sample from batch 91368-001, generously supplied by Miss B. Stearns of the Squibb Institute for Medical Research.

B. Osmotic Experiments

The net flow of water produced by a concentration difference of solute across the membrane was measured with a cell slightly modified from that described by Cass and Finkelstein (1967) (Fig. 3); the solutes were NaCl, urea, ethylene glycol, glycerol, glucose, and sucrose. The cell consisted of joined lengths of polyethylene tubing terminated at one end by a Hamilton microliter syringe, No. 7101, and contained a Ag/AgCl electrode that terminated approximately 3 mm from the open end. The other end of the electrode was brought out through the wall of the polyethylene tube, and this region was tightly sealed with epoxy cement. The lipid film, when formed across the open end (area 1.02mm^2), closed off the volume of the tube from the external solution. The syringe controlled this inner volume, and thus the degree of curvature of the film. The pattern of light from the fully black film was dependent

on this curvature and was sensitive to syringe displacements of less than $0.005 \mu\text{l}$. The tubing was mounted in a 100 ml thermostated ($\pm 0.02^\circ\text{C}$) stainless steel box,

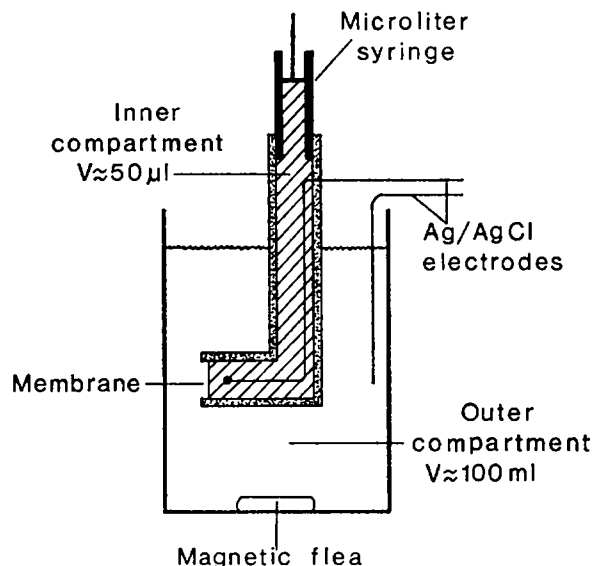


FIGURE 3. Schematic drawing of cell used for osmotic experiments.

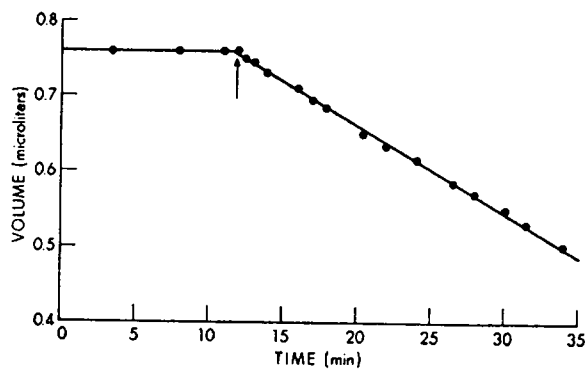


FIGURE 4. Volume flow of water across a nystatin-treated membrane produced by a concentration difference of glucose. The membrane was formed in 100 mM NaCl, 5 mM sodium phosphate (pH 7), $2.5 \mu\text{g/ml}$ nystatin. The membrane attained a steady resistance of $9 \times 10^8 \Omega$ ($90 \Omega\text{cm}^2$). At the arrow glucose was added to the outer chamber to a concentration of 209 mM. Area of membrane = 1.02 mm^2 . From the slope of the line and the film area, P_f is found from equation (3) to be $5.02 \times 10^{-8} \text{ cm/sec}$. (Subtracting $0.2 \times 10^{-8} \text{ cm/sec}$ for the P_f of the unmodified membrane and normalizing to a resistance of $100 \Omega\text{cm}^2$, we obtain $4.3 \times 10^{-8} \text{ cm/sec}$ as the normalized P_f of the nystatin regions.) Temperature = $25^\circ\text{C} \pm 0.02^\circ\text{C}$.

which contained a Ag/AgCl electrode; a magnetic flea continuously stirred the solution in the box during an experiment. The electrical resistance of the membrane was monitored throughout an experiment as described for the tracer experiments.

Membranes were formed at 25°C in either 100 mM NaCl, 5 mM sodium phosphate (pH 7) or 10 mM NaCl, 0.5 mM sodium phosphate (pH 7); the solutions also contained the appropriate amount of nystatin or amphotericin B. A typical experiment proceeded as follows: after the membrane had become completely black, stirring of the solution in the box was begun. When the membrane resistance had attained a constant value, a small volume of concentrated solution of the desired solute was added to the box; mixing was completed within 15 sec. The film began to bulge inward as water left the inner cell. By movement of the syringe to keep the pattern of light unchanged, and thus the area constant, the volume as a function of time was obtained. The data from a typical experiment are shown in Fig. 4. It was often possible to make two or three changes of concentration on one film. Volume flow was directly proportional to osmotic pressure over the range 2 to 16 atmospheres (Δc 's from 0.1 to 0.7 osmolar).

II. THEORY

A. Tracer Measurements

The flux, Φ_i^* , of labeled species "*i*" across a membrane separating identical solutions, is given by:

$$\Phi_i^* = -(P_d)_i A (c_{i_2}^* - c_{i_1}^*) \quad (1)$$

where A is the area of the membrane, $c_{i_2}^*$ and $c_{i_1}^*$ are the concentrations of i^* in solutions (2) and (1), respectively, and $(P_d)_i$ is the permeability coefficient of the membrane for species i . Equation (1) can be derived from a variety of assumptions but is most simply viewed as a definition of the permeability coefficient $(P_d)_i$. (The linear dependence of flux on the concentration difference of i^* is invariably observed experimentally in studies of membrane transport, provided the concentration of i^* is small compared to that of i .) From the slope of $c_{i_1}^*$ vs. time and the volume of side 1, we obtain Φ_i^* , and then calculate $(P_d)_i$ from (1). Because of the small fluxes involved in our experiments, $(c_{i_2}^* - c_{i_1}^*) \approx c_{i_2}^*$ throughout the course of an experiment.

B. Osmotic Measurements

1. IMPERMEANT SOLUTE

It can readily be shown (Cass and Finkelstein, 1967) that the flux, Φ_w , of water (in moles per unit time) across a membrane of area A in the presence of a concentration difference of a *completely impermeant* solute, i , is given by:

$$\Phi_w = -P_f A [\phi_{i_2} c_{i_2} - \phi_{i_1} c_{i_1}] \quad (2)$$

where ϕ_i is the osmotic coefficient of solute i , the subscripts 1 and 2 refer to sides 1 and 2 of the membrane, and P_f is the filtration (or osmotic) permeability coefficient of the membrane. For the solutes and the range of concen-

trations used in the present experiments, $\phi_{i_2} \approx \phi_{i_1} \equiv \phi_i$. Defining

$$c_{i_2} - c_{i_1} \equiv \Delta c_i$$

equation (2) is then rewritten as:

$$\begin{aligned} \Phi_w &= -P_f A \Delta c_i \times \phi_i \quad (\text{nonelectrolytes}) \\ \Phi_w &= -P_f A \Delta c_{\text{NaCl}} \times 2 \times \phi_{\text{NaCl}} \quad (\text{NaCl}) \end{aligned} \quad (3)$$

From the slope of the syringe volume (V) vs. time and the partial molar volume of water, Φ_w is readily obtained, and P_f is then calculated from (3). In principle P_f and $(P_d)_w$ are independent quantities; their relative magnitudes have been used to infer the mechanism by which water crosses a membrane and thus to infer something about the nature of the membrane itself (see Discussion).

2. PERMEANT SOLUTE

If a membrane is permeable to solute i , the volume flow, $(J_v)_i$, observed for a given $\phi_i \times \Delta c_i$ will be less than that observed if i is a completely impermeant solute (Staverman, 1951; Kedem and Katchalsky, 1958). In fact, if J_v is the volume flow observed for a given gradient of an impermeant solute and if $(J_v)_i$ is the volume flow observed for the same gradient of permeant solute i , then the reflection coefficient, σ_i , of solute i is defined by:

$$\sigma_i \equiv \frac{(J_v)_i}{J_v} \quad (4)$$

(Staverman, 1951, Kedem and Katchalsky, 1958).

σ_i , like $(P_d)_i$, is a measure of the membrane's permeability to species i , but the two quantities are in principle completely independent. σ is a measure of the degree of interaction between solute and water as they traverse the membrane, and again it is possible to infer mechanism of transport and membrane structure from a proper comparison of σ values with P_d values (see Discussion).

To summarize: two water permeability coefficients, $(P_d)_w$ and P_f , are determined from tracer measurements and osmotic measurements, respectively. Similarly, for each solute i two permeability coefficients, $(P_d)_i$ and σ_i , are determined. The experiments reported below measure these quantities for water and several solutes. From these results we shall draw certain conclusions about the nature of a thin lipid membrane treated with nystatin or amphotericin B.

III. RESULTS

A. *Tracer Experiments*

1. UNSTIRRED LAYERS In order to determine the true values of P_d for the various species tested, we must consider the contribution of unstirred layers to the tracer permeability data.¹ This is necessary, because there will always exist regions of incomplete mixing near the membrane, despite continuous stirring on both sides (Schulman and Teorell, 1938; Peterson and Gregor, 1959). The actual concentration profiles in these regions are exceedingly complex and virtually impossible to calculate explicitly for most cases. It is possible, however, to idealize this situation, and imagine complete mixing on both sides of the membrane up to distances δ_1 and δ_2 from each interface, with no mixing closer to the membrane. In these "unstirred layers", only diffusion is assumed to occur. With these assumptions it is easily shown that:

$$\frac{1}{[(P_d)_i]_{\text{obs}}} = \frac{\delta}{D_i} + \frac{1}{(P_d)_i} \quad (5)$$

where $(P_d)_i$ is the *actual* permeability coefficient of the membrane for the species i , and the quantity of interest, $[(P_d)_i]_{\text{obs}}$ is the permeability coefficient observed experimentally, D_i is the free diffusion coefficient of i in solution, and $\delta = \delta_1 + \delta_2$. Rewriting (5) we have:

$$[(P_d)_i]_{\text{obs}} = (P_d)_i \left(\frac{1}{1 + \frac{(P_d)_i}{D_i/\delta}} \right) \quad (6)$$

The effect of the unstirred layers on $[(P_d)_i]_{\text{obs}}$ is dependent on the ratio of $(P_d)_i$ to D_i/δ . If the membrane is not very permeable to i , that is, if $(P_d)_i \ll D_i/\delta$, then (6) becomes:

$$[(P_d)_i]_{\text{obs}} \approx (P_d)_i \left(\text{for } (P_d)_i \ll \frac{D_i}{\delta} \right) \quad (6a)$$

On the other hand, if the membrane is highly permeable to i , (6) reduces to

$$[(P_d)_i]_{\text{obs}} \approx \frac{D_i}{\delta} \left(\text{for } (P_d)_i \gg \frac{D_i}{\delta} \right) \quad (6b)$$

¹ For the flow rates and Δc 's in our osmotic experiments, it can be shown theoretically, and is an experimental fact, that if the osmotic difference is produced by an *impermeant solute*, the unstirred layer correction is trivial in determining P_f (Dainty, 1963; Cass and Finkelstein, 1967). If the osmotic difference is produced by a permeant solute, however, this is not necessarily the case, as we will discuss later.

and the observed P_d is the permeability of the unstirred layer region, independent of the actual membrane permeability.

$(P_d)_i$ can be calculated from $[(P_d)_i]_{obs}$ using equation (6) provided that δ can be determined. We measured δ by taking advantage of the permeability properties of the unmodified thin lipid membrane. It is our working assumption that for any molecule that is more soluble in hydrocarbon than in water, the value of P_d will be so large that equation (6 *b*) will hold (see Lauger et al., 1967, for a typical calculation).² Thus, δ is directly measurable.

We chose butanol as a molecule that satisfies the above solubility requirements and obtained, using *n*-butanol-1-C¹⁴,

$$[(P_d)_{butanol}]_{obs} = 5.9 \times 10^{-4} \text{ cm/sec}$$

Substituting this and the diffusion coefficient of butanol in water at 25°C [1×10^{-5} cm²/sec (Lyons and Sandquist, 1953)] into (6 *b*) we obtain

$$\delta = 170 \mu$$

the thickness of the unstirred layer in our present experiments.

We are confident in this result for three reasons: first, the same result was obtained whether the membrane was treated with antibiotic or not; this we would expect, since $[(P_d)_{butanol}]_{obs}$ should be independent of the permeability of the membrane. Second, since we feel that the unstirred layer primarily resides in the channel behind the membrane (Cass and Finkelstein, 1967), the value of 170 μ agrees well with the measured channel thickness of 150 μ .³ Third, and most convincing, is the dependence of $[(P_d)_{butanol}]_{obs}$ on the viscosity of the aqueous solutions. From diffusion theory, D should vary inversely with viscosity; therefore, if butanol permeation is completely unstirred layer limited, then from equation (6 *b*), $[(P_d)_{butanol}]_{obs}$ should decrease in a more viscous medium. We measured P_d of butanol for a membrane in a solution containing 2 M glucose in addition to 100 mM NaCl, 5 mM sodium phosphate. The viscosity of this solution is 2.8 times that of 100 mM NaCl, and, *mirabile dictu*, the measured P_d in this solution was 1/2.8 that in 100 mM NaCl.

For all molecules except water, we worked within a range of values of P_d where the unstirred layer correction was usually less than 10%. The unstirred layer correction was crucial, however, for an accurate determination of P_d for water (compare Figs. 5 a and 5 b). The inherent difficulty in determining water permeability increases caused by the antibiotics is that $(P_d)_w$

² Thus, we feel that the values of P_d reported by Bean et al. (1968) for such lipophilic solutes as indole and indole-3-ethanol are really just measures of the unstirred layers in those experiments.

³ This is larger than the 125 μ thickness of the partition, because polyethylene flows when a hole is made in it with a hot needle, thus thickening the partition around the hole.

of the *unmodified* membrane can be so large that unstirred layer corrections are already significant. (See Cass and Finkelstein, 1967, and Everitt et al., 1969, for a discussion of this point.) In our experiments, increases in $(P_d)_w$ produced by the antibiotics were readily apparent only because the lipid

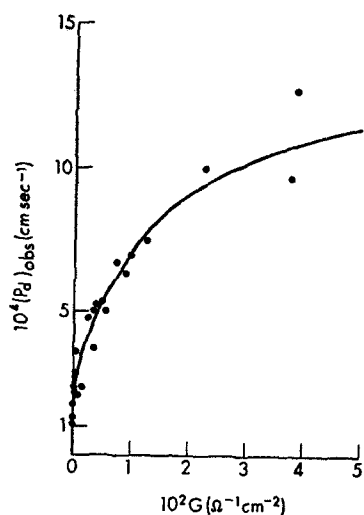


FIGURE 5 a. The *observed* values of $(P_d)_{\text{water}}$ (uncorrected for unstirred layers) as a function of conductance for nystatin-treated membranes. All experiments were performed at $25^\circ\text{C} \pm 1^\circ\text{C}$ in 100 mM NaCl, 5 mM sodium phosphate (pH 7), 0–5 $\mu\text{g}/\text{ml}$ nystatin.

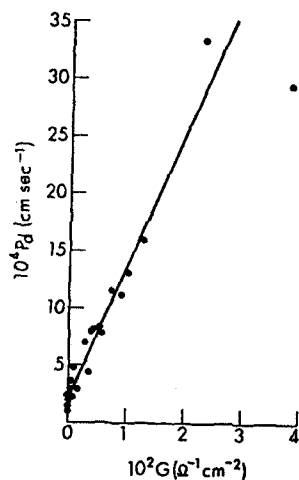


FIGURE 5 b. The *actual* values of $(P_d)_{\text{water}}$ as a function of conductance for nystatin-treated membranes. These are the same data as are given in Fig. 5 a; each point has been corrected for unstirred layers using equation (6) with $D = 2.4 \times 10^{-5} \text{ cm}^2/\text{sec}$ and $\delta = 0.017 \text{ cm}$. (One corrected point from Fig. 5 a is off-scale in this figure.)

mixture was judiciously chosen to yield the very low value for $(P_d)_w$ of the unmodified membrane of $2 \times 10^{-4} \text{ cm}/\text{sec}$. This low value of $(P_d)_w$ was achieved by the use of ox-brain white matter lipids, which in themselves have a relatively low water permeability (Finkelstein and Cass, 1967) and by the addition of a large amount of cholesterol to the membrane-forming solution, which considerably decreases the water permeability (Finkelstein and Cass, 1967).

2. DEPENDENCE OF P_d ON MEMBRANE CONDUCTANCE Before giving the values of P_d for the various species, we wish to make an important general point: at a given salt concentration, pH, and temperature, *the values of P_d for all solutes and water increase linearly with the membrane conductance*, which, in turn, is controlled by the antibiotic concentration (see, for example, Figs. 5 b and 6). It is therefore possible to normalize any value of P_d to that of a membrane with any arbitrary resistance. For analysis our tabulated results are normalized to a resistance of $100 \Omega\text{cm}^2$ ($G = 10^{-2} \Omega^{-1}\text{cm}^{-2}$) in 0.1 M NaCl. At

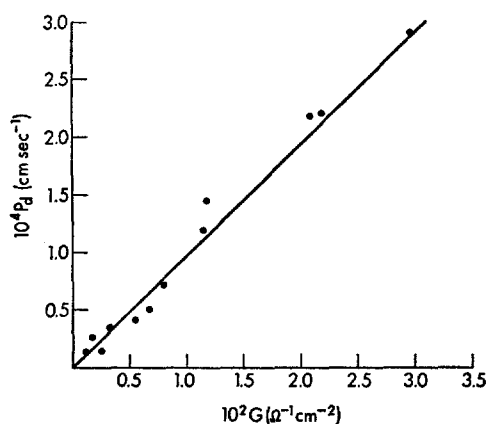


FIGURE 6. $(P_d)_{\text{urea}}$ as a function of conductance for nystatin-treated membranes. (All points have been corrected for unstirred layers using equation (6) with $D = 1.4 \times 10^{-5} \text{ cm}^2/\text{sec}$ and $\delta = 0.017 \text{ cm}$; only for those points at which $P_d > 1 \times 10^{-4} \text{ cm}/\text{sec}$ is this correction greater than 10%.) All experiments were performed at $25^\circ\text{C} \pm 1^\circ\text{C}$ in 100 mM NaCl, 5 mM sodium phosphate (pH 7), 0–5 $\mu\text{g}/\text{ml}$ nystatin. Some solutions contained, in addition, either 10 mM or 100 mM urea.

conductances below $10^{-4} \Omega^{-1}\text{cm}^{-2}$, the values of P_d for the solutes tested were below the detectable level ($2 \times 10^{-6} \text{ cm}/\text{sec}$); therefore, we can set equal to 0 both conductance and $(P_d)_{\text{solute}}$ of the unmodified membrane (actually $G \approx 10^{-8} \Omega^{-1}\text{cm}^{-2}$). Only in the case of water is the value of P_d of the unmodified membrane significant; namely, $2 \times 10^{-4} \text{ cm}/\text{sec}$. Thus, it is gratifying that in Fig. 5 b the plot of $(P_d)_w$ vs. conductance passes through this value at 0 conductance.

Because membrane conductance depends on a large power of the antibiotic concentration (Cass et al., 1970) and because these antibiotics slowly lose activity upon standing in aqueous solution (Dutcher et al., 1955), it is not unusual for membrane conductances to differ by a factor of 4 or more in the presence of identical concentrations of polyene. Consequently, it is imperative to relate water and solute permeability to membrane conductance rather than to antibiotic concentration.

3. VALUES OF P_d In the first column of Table I we tabulate the normalized values of P_d for water, urea, thiourea, ethylene glycol, and glycerol for nystatin-treated membranes.⁴ We see that permeability of these small molecules decreases sharply with increasing molecular size. Thus, compared to water, whose P_d is 12×10^{-4} cm/sec, urea and thiourea are only one-thirteenth as permeant; ethylene glycol is approximately one-twenty-sixth as permeant; and glycerol is only one one-hundredth as permeant as water.⁵ In the third column of Table I are tabulated the normalized values of

TABLE I
PERMEABILITY OF THIN LIPID MEMBRANES IN THE
PRESENCE OF NYSTATIN OR AMPHOTERICIN B

	Nystatin		Amphotericin B	
	$P_d(\text{cm/sec})$	σ	$P_d(\text{cm/sec})$	σ
Water	12.0×10^{-4}	0	6.0×10^{-4}	0
Urea	0.95×10^{-4}	0.55	0.68×10^{-4}	0.57
Thiourea	0.95×10^{-4}	—	—	—
Ethylene glycol	0.45×10^{-4}	0.67	—	—
Glycerol	0.115×10^{-4}	0.78	0.075×10^{-4}	—
Glucose	—	1.0	—	1.0
Sucrose	—	1.0	—	—
NaCl	—	1.0	—	1.0
	$P_f = 40 \times 10^{-4}$ cm/sec		$P_f = 18 \times 10^{-4}$ cm/sec	
	$\frac{P_f}{(P_d)_{\text{water}}} = 3.3$		$\frac{P_f}{(P_d)_{\text{water}}} = 3.0$	

All P_d 's and P_f 's are values normalized for membranes of resistance $100 \Omega\text{cm}^2$ in 0.1 M NaCl.

P_d for amphotericin B—treated membranes.⁴ The *relative* selectivity among neutral species is the same in both amphotericin B- and nystatin-treated membranes; however, for membranes at the same conductance the *absolute* values of P_d are approximately 50% smaller with amphotericin B.

We observed no effect of the absolute concentration of solute on the normalized value of P_d . For example, P_d for urea was the same when either 0 mM, 10 mM, or 100 mM urea was present on both sides of the membrane.

⁴ In the value given for water, we have subtracted the P_d of the unmodified membrane.

⁵ There is the possibility in using THO that one is measuring proton flux rather than water flux. To check this, we measured $(P_d)_w$ at pH 5.4 instead of pH 7.0. If proton conduction were important, $(P_d)_w$ should have increased enormously. In fact, there was a small (~50%) decrease in $(P_d)_w$. (A similar decrease in $(P_d)_{\text{urea}}$ was also observed at pH 4.2.) Thus, THO appears to be acting as a true tracer of H_2O .

B. Osmotic Experiments

1. DEPENDENCE OF P_f ON MEMBRANE CONDUCTANCE P_f , like P_d , increases linearly with membrane conductance (Fig. 7).⁶ Thus, we can also normalize the values of P_f to a conductance of $10^{-2} \Omega^{-1} \text{cm}^{-2}$ in 100 mM NaCl and obtain a value of $40 \times 10^{-4} \text{cm/sec}$ for a nystatin-treated membrane.⁷ (P_f determined in 10 mM NaCl also increases linearly with membrane conductance. However, at any value of P_f ,⁷ the conductance of a membrane in 10 mM NaCl is one-fortieth the conductance of a membrane in 100 mM NaCl.

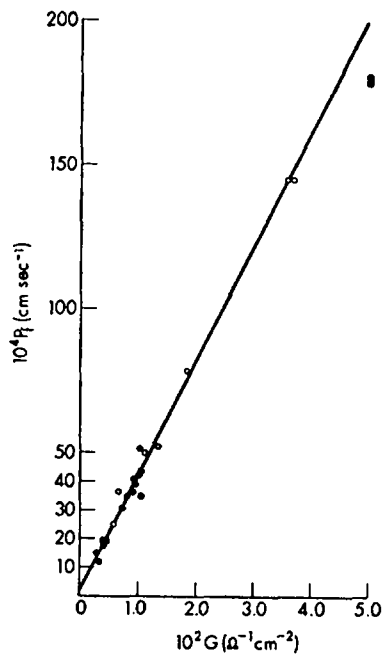


FIGURE 7. P_f as a function of conductance for nystatin-treated membranes. All membranes were formed at $25^\circ\text{C} \pm 0.02^\circ\text{C}$ in 100 mM NaCl, 5 mM sodium phosphate (pH 7), 1–5 $\mu\text{g/ml}$ nystatin. Osmotic gradients produced with NaCl (●) or glucose (○).

See A-5 of the Discussion.) We note that at 0 conductance, P_f extrapolates to $2 \times 10^{-4} \text{cm/sec}$, the same value as $(P_d)_w$. Thus, for the unmodified membrane, $P_f/(P_d)_w = 1$, a result previously reported (Cass and Finkelstein, 1967), whereas for the nystatin-treated membrane, $P_f/(P_d)_w = 3.3$. For the amphotericin B treated-membrane, the normalized value of P_f is $18 \times 10^{-4} \text{cm/sec}$, but since the normalized value of $(P_d)_w$ is 6×10^{-4} , $P_f/(P_d)_w = 3.0$, in good agreement with the ratio obtained for the nystatin-treated membrane.

2. VALUES OF σ For a given osmotic gradient at a given conductance, NaCl, glucose, or sucrose induced the same rate of volume flow, and this

⁶ There was, as expected (Cass et al., 1970), a large increase in conductance following addition of NaCl as test solute. The conductance used in our calculations was the value *prior* to addition of the salt gradient.

⁷ We have subtracted the P_f of the unmodified membrane which is $2 \times 10^{-4} \text{cm/sec}$.

rate was greater than those induced by urea, ethylene glycol, or glycerol. On this basis, we can assign a value of 1 for σ of glucose, sucrose, and NaCl, and it was from the flow rates obtained with these solutes that P_f was calculated. The σ for another solute is then the ratio of the flow rate obtained with that solute to the rate produced at the same conductance by the same osmolar difference of glucose, sucrose, or NaCl (see equation 4).⁸ This was a straight-

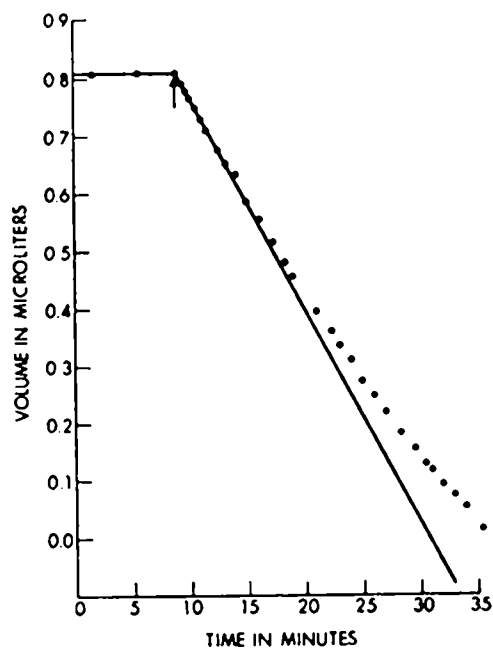


FIGURE 8. Volume flow across a nystatin-treated membrane for a concentration difference of ethylene glycol. The membrane was formed in 10 mM NaCl, 0.5 mM sodium phosphate (pH 7), 2.8 $\mu\text{g}/\text{ml}$ nystatin. The membrane attained a steady resistance of $1.9 \times 10^4 \Omega$ ($1.9 \times 10^4 \Omega\text{cm}^2$). At the arrow ethylene glycol was added to the outer compartment to a concentration of 493 mM. Membrane area = 1.02 mm²; temperature = 25°C \pm 0.02°C. Note the slowing of the rate with time in contrast to the steady flow rate obtained with glucose in Fig. 4.

forward procedure for glycerol, a solute for which the rate of flow was constant over time. A problem arose, however, when urea or ethylene glycol was the test solute.

Fig. 8 is a typical plot of volume vs. time obtained with ethylene glycol

⁸ Since σ of all solutes tested is 1 for the unmodified membrane (Finkelstein and Cass, 1968) and since, as we shall argue in the Discussion, most of the membrane remains unmodified even in the presence of antibiotic, (J_s), and J_s in equation 4 are calculated by subtraction of flow rate produced on the unmodified membrane from observed flow rates. Thus, the σ 's that we calculate are the σ 's in the antibiotic-modified region of the membrane.

as the test solute. Note that the rate of flow continuously decreases with time. Similar curves were obtained with urea. This is in contrast to Fig. 4, obtained with glucose, in which volume vs. time is linear. We believe that the non-linearity is caused by changes with time in solute concentration at the inner membrane interface, because of diffusion of these permeant solutes through the membrane.⁹ The solute concentration at the outer interface is maintained by stirring, but because of the inaccessibility of the solution in the tube to stirring, the concentration of solute at the inner interface increases as solute diffuses through the membrane. The result is that the effective Δc across the membrane continuously decreases and, therefore, the rate of volume flow decreases.

This interpretation is supported by three sets of data: first, nonlinearity did not occur with virtually impermeant solutes—glucose, sucrose, and NaCl—nor with the poorly permeable glycerol; second, for a given solute, nonlinearity was more pronounced the higher the membrane conductance (and, hence, the higher the P_d); and third, at a given conductance, nonlinearity was more pronounced for the more permeable urea than for the less permeable ethylene glycol.

It is very difficult to derive theoretically the shape of the volume vs. time curve, because diffusion of solute within the tube is complicated both by mass flow of solvent and by convective mixing due to density gradients. If we consider only diffusion and neglect these complicating factors, the problem of calculating Δc across the membrane at any time is solvable (Crank, 1956). This analysis shows that the rate of change of Δc (and, hence, the rate of change of volume flow) is strongly dependent on the value of P_d , as indeed observed in our experiments.

The flow rate used to calculate σ was the initial slope in the volume vs. time curve; that is, we took the flow rate produced by the applied Δc of solute, before the concentrations at the boundaries had changed significantly. The results for nystatin-treated membranes are tabulated in the second column of Table I. The σ 's thus calculated were independent of conductance over the entire experimental range, indicating that this procedure gave accurate determinations of σ ($\pm 10\%$).

σ increases from 0.55 for urea to 1 for glucose, sucrose, and NaCl. This trend parallels the trend in the P_d data. That is, the smaller the σ , the larger the P_d . Among the permeant solutes, only urea was tested on amphotericin B-treated membranes; a σ value of 0.57 was obtained, in excellent agreement with the value obtained on nystatin-treated membranes.

⁹ The constant membrane conductance during the course of the experiment demonstrates that this nonlinearity is not the result of a direct effect of these solutes on membrane permeability.

IV. DISCUSSION

A. *The Nature of a Membrane Treated with Nystatin or Amphotericin B*

1. THE EXISTENCE OF AQUEOUS PORES It is very instructive to compare the permeability to water and nonelectrolytes of an unmodified thin lipid membrane with one treated with either nystatin or amphotericin B. For the former, $P_f/(P_d)_w = 1$ and for all hydrophilic solutes $\sigma = 1$ and P_d 's are very small. For the polyene-treated film, on the other hand, $P_f/(P_d)_w \approx 3$, and for all hydrophilic solutes, σ increases with increasing molecular size while P_d decreases. Also, the absolute values of $(P_d)_w$, $(P_d)_{\text{solute}}$, and P_f increase linearly with membrane conductance. The available data are consistent with the view that molecules cross unmodified membranes by dissolving in the hydrocarbon portion of the membrane and then diffusing through this region (Finkelstein and Cass, 1968). What can be said of the mechanism by which molecules traverse nystatin- or amphotericin B-treated membranes?

Because $P_f/(P_d)_w$ is significantly greater than 1 (namely, 3) and the σ values for hydrophilic solutes increase with increasing molecular size, we conclude that *nystatin and amphotericin B create aqueous pores in thin lipid membranes*. Certainly, these criteria have traditionally been accepted by physiologists as evidence for the existence of pores. For if $P_f/(P_d)_w$ is significantly greater than 1, water molecules move in a cooperative manner during osmosis; they "see" each other. Similarly, σ values significantly less than 1 mean that solutes interact with water molecules when they traverse the membrane;¹⁰ they "see" water. These facts, however, can *in principle* also be made consistent with a "liquid membrane" containing a significant amount of water in its liquid phase; and indeed we are not unaware that values of $P_f/(P_d)_w$ greater than 1 and σ values significantly less than 1 can be obtained both theoretically and experimentally in water-rich liquid membranes (Sidel and Hoffman, 1961, 1963; Thau et al., 1966). However, if nystatin and amphotericin B were somehow increasing the over-all water content of the membrane, then as water content increased with antibiotic concentration, $P_f/(P_d)_w$ would continuously increase, while σ 's would continuously decrease. But we find that $P_f/(P_d)_w$ and σ_{solute} are *independent* of antibiotic concentration (membrane conductance). The simplest interpretation of our results, therefore, is that nystatin and amphotericin B do not affect the over-all nature of the film, but rather introduce local modifications; the more antibiotic added, the more numerous these modifications; and the simplest picture of these modifica-

¹⁰ The σ 's for urea, ethylene glycol, and glycerol are much lower than $\left(1 - \frac{(P_d)_{\text{solute}}}{P_f} \cdot \frac{\bar{V}_{\text{solute}}}{\bar{V}_{\text{water}}}\right)$, the values σ would have if these solutes crossed the membrane through regions different from those crossed by water.

tions is that they are aqueous pores. By an aqueous pore we mean a local modification of such high water content that any molecule, including water itself, passing through it interacts significantly with water.

2. THE SIZE OF THE PORES It is a popular activity among some physiologists to calculate the "equivalent pore radius" for porous membranes. Such calculations can be made from the value of $P_f/(P_d)_w$ (Paganelli and Solomon, 1957) or from either the P_d 's or σ 's by "restricted diffusion theory" (Renkin, 1954). We do not place great emphasis on the values so obtained, however, because the assumptions underlying these calculations are inappropriate on the molecular level. As a criterion of pore size, we feel it is sufficient to note that σ for glycerol is approximately 0.8 and σ of glucose is 1; i.e., the membrane is quite tight to glycerol and essentially impermeable to glucose. On this basis, a pore radius of approximately 4 Å can be assigned. (Approximately the same radius is obtained from calculations based on the $P_f/(P_d)_w$ value or from restricted diffusion theory using our P_d or σ data.) In assigning this pore radius, we mean that molecules of Stokes-Einstein radius larger than 4 Å are effectively excluded from the membrane.

We recognize that the ability of a small hydrophilic solute to enter and pass through an aqueous pore is dependent not only on its Stokes-Einstein radius, but also on such interactions between solute and pore as hydrogen bonding, dipole-dipole forces, and dipole-induced dipole forces. Nevertheless, we find that the permeability of small hydrophilic solutes through pores created by nystatin or amphotericin B is (to a first approximation) inversely correlated with their Stokes-Einstein radius. We also recognize that a pore considerably larger than 4 Å in radius might still effectively exclude molecules of this size because of these other factors.

3. CORRELATION BETWEEN P_d AND CONDUCTANCE We wish to draw particular attention to the linear relation between the values of P_d for hydrophilic solutes (including water) and electrical conductance. This implies that a channel allowing permeation of water and small solutes also acts as a conduction pathway for ions. This is particularly convincing because conductance is not a linear function of antibiotic concentration, but rather depends on a large power of this concentration (Cass et al., 1970). If one class of channels conducts ions and another class conducts water and small solutes, we must assume that the number of each of these two types of channels follows the same unusual power dependence on antibiotic concentration; this is unlikely.

4. AREA OCCUPIED BY PORES Implicit in our discussion and also explicit in our calculations of P_d 's, P_f , and σ 's has been the assumption that a relatively small area is occupied by pores even in a high conductance membrane. The following calculation justifies this assumption: For a nystatin-treated membrane of conductance $10^{-2} \Omega^{-1}\text{cm}^{-2}$, $(P_d)_w = 12 \times 10^{-4} \text{ cm/sec}$. If we

assume that water within a pore has approximately the same diffusion coefficient as in free solution ($D = 2.4 \times 10^{-5}$ cm²/sec) and the membrane thickness, δ , is approximately 50 Å (Hanai et al., 1964; Tien, 1967), then the area, A , occupied by the pores per cm² of membrane surface is given by:

$$A = \frac{(P_d)_w \delta}{D} = \frac{(12 \times 10^{-4})(50 \times 10^{-8})}{2.4 \times 10^{-5}} = 25 \times 10^{-6} \text{ cm}^2$$

Thus, less than 0.01% of membrane area is occupied by pores, even if antibiotic has reduced the membrane resistance from $10^8 \Omega\text{cm}^2$ to $10^2 \Omega\text{cm}^2$. Hence, the nystatin- or amphotericin B-treated membrane is a mosaic structure, containing a small number of aqueous pores (through which water, small hydrophilic solutes, and ions pass) in a vast expanse of unmodified membrane (through which lipophilic molecules and some water pass). This is not unlike the picture of plasma membranes that is generally accepted by physiologists and which has its historical roots in the work of Overton (1895) and Collander (1937).

5. THE EFFECT OF SALT CONCENTRATION ON THE PORES In order to compare values of P_d and P_f at different salt concentrations, it is necessary to understand the effect of changing salt concentration on a polyene-treated membrane. The following experiment illustrates the effect: $(P_d)_w$ of a nystatin-treated membrane was measured in 0.01 M NaCl. Midway through the experiment, the NaCl concentration on both sides was increased to 0.1 M NaCl. The conductance immediately increased 40-fold (see also Fig. 3 of Cass et al., 1970), but the flux of THO remained the same; that is, $(P_d)_w$ did not change. It appears that changing NaCl concentration merely changes the conductance per pore, but not the number or structure of the pores. Consistent with this interpretation are the observations that the ratio of P_f to $(P_d)_w$ is the same (namely, 3.3) when measured in either 0.01 M NaCl or 0.1 M NaCl, and at a given value of P_f , the conductance in 0.1 M NaCl is 40-fold larger than the conductance in 0.01 M NaCl (B-1 of Results).

6. COMPARISON OF NYSTATIN AND AMPHOTERICIN B As shown in Table I, nystatin- and amphotericin B-treated membranes have almost the same selectivity, but the absolute values of P_f and P_d are approximately twice as large in nystatin-treated membranes. Nystatin- and amphotericin B-created pores therefore appear to have the same permeability to water and small hydrophilic solutes, but the conductance (ion permeability) of an amphotericin B-created pore is approximately twice that of a nystatin-created pore. In other words, at a given conductance an amphotericin B-treated membrane has half as many pores as a nystatin-treated membrane.

7. COMPARISON TO THE RED CELL MEMBRANE Although the nystatin- or amphotericin B-treated membrane is a useful general model of plasma mem-

branes (see section 4), it is particularly striking as a model for the human red cell membrane. In Table II we compare $P_f/(P_a)_w$ and σ 's for the red cell membrane and the nystatin-treated thin lipid membrane. The agreement is remarkable. In addition, both membranes are anion selective. Furthermore, reagents that modify amino groups reduce the anion permeability of the red cell (Schnell and Passow, 1969), and *N*-acetylation and *N*-succinylation reduce the activity of nystatin (Cass et al., 1970). All this is probably coincidental. Nevertheless, it is possible that these similarities are more than phenomenological, and it therefore might not be completely fruitless to attempt to extract a polyene or polyene-like molecule from red cell membranes.

TABLE II
COMPARISON OF THE PERMEABILITY OF NYSTATIN-TREATED
THIN LIPID MEMBRANE WITH THE PERMEABILITY OF
HUMAN RED BLOOD CELL MEMBRANE

	Nystatin-treated membrane	Red blood cell membrane
σ Urea	0.55	0.62
σ Ethylene glycol	0.67	0.63
σ Glycerol	0.78	0.88
$P_f/(P_a)_{\text{water}}$	3.3	2.5

Data for human red cell membrane taken from Goldstein and Solomon (1960) and Paganelli and Solomon (1957).

B. Comparison with Previous Work

Aside from an isolated observation by Lippe (1968) on amphotericin B-treated membranes, and some measurements of P_f and σ 's by Finkelstein and Cass (1968) on nystatin-treated membranes, the only other data we know on the permeability to water and nonelectrolytes of polyene antibiotic-treated thin lipid membranes are those of Andreoli et al. (1969) on amphotericin B-treated films. We shall now compare our present results with those of Andreoli et al.

At the outset we wish to emphasize that *qualitatively* there is complete agreement between the data they obtained with amphotericin B and those we summarize in Table I. Thus, both agree that amphotericin B increases the permeability of the films to water and small hydrophilic solutes and that the permeability to these solutes decreases with increasing molecular size. *Quantitatively*, however, there are striking differences between their results and ours: First, ratios of P_a for any two solutes are much smaller in their experiments than in ours. For example, they report a ratio of $(P_a)_w$ to $(P_a)_{\text{urea}}$ of 1.8, whereas we obtain a ratio of 14. Second, all their nonunity σ values

are considerably lower than ours. Thus, they report $\sigma_{\text{urea}} = 0.08$, whereas we obtain 0.57. Third, they report $P_f/(P_d)_w = 30$, whereas we obtain 3. These discrepancies could reflect differences in the lipid composition of the membranes; we believe, however, that they are caused by unstirred layers. As Andreoli et al. (1969) state, “. . . the presence of unstirred layers in the aqueous phases adjacent to the membranes might have reduced the actual value of P_{D_w} and, to a lesser extent, of P_{D_i} for the more permeable solutes (i.e., urea, acetamide, or glycerol.)” We agree with the authors, but feel that the unstirred layers present a much greater problem than they suggest.

Considering first their tracer data, we find that for amphotericin B-treated membranes they obtain $(P_d)_w = 18 \times 10^{-4}$ cm/sec. In our experiments, the largest possible value obtainable is 14×10^{-4} cm/sec, because of the unstirred layer problem. Since their experimental arrangement for stirring was not significantly different from ours, we conclude that the value of 18×10^{-4} cm/sec is the water permeability of the unstirred layer and that the actual value of $(P_d)_w$ for their membranes is many fold larger. On this basis we calculate from equation 6 *b* a thickness for their unstirred layer of 130μ , in reasonable agreement with 170μ in our experiments. Substituting this value for δ into equation 6 *b*, we find that their P_d values for acetamide and urea are also almost completely unstirred layer limited. Thus, the ratios (taken between any two) of $(P_d)_w$, $(P_d)_{\text{urea}}$, and $(P_d)_{\text{acetamide}}$ are approximately the same as the corresponding ratios of the free diffusion coefficients of these molecules. The only P_d value which can be used for comparison with our own is that of glycerol. When corrected for unstirred layers, it turns out to be 7×10^{-4} cm/sec as compared to our value of 0.075×10^{-4} cm/sec obtained at a conductance of $10^{-2} \Omega^{-1} \text{cm}^{-2}$ in 0.1 M NaCl. On this basis, we estimate that Andreoli et al. measured water and solute permeabilities on membranes whose conductances would have been $\approx 1 \Omega^{-1} \text{cm}^{-2}$ in 0.1 M NaCl;¹¹ the *highest* conductance at which we made P_d measurements was $3 \times 10^{-2} \Omega^{-1} \text{cm}^{-2}$.¹² To summarize then, it appears that the values of P_d for water, acetamide, and urea as measured by Andreoli et al., are almost completely limited by unstirred layers. Because of this underestimation of $(P_d)_w$, their ratio of 30 for P_f to $(P_d)_w$ is much too large.

Concerning their osmotic experiments on amphotericin B-treated membranes, we feel that the small σ 's they obtained for urea, acetamide, and glycerol resulted from a reduction of effective Δc across the membrane caused by the large permeability of the high conductance membranes with which they were working (see B-2 of Results). Similarly low values of σ originally

¹¹ Andreoli et al. performed their experiments in salt-free solutions and did not measure conductance.

¹² It is possible that the small finite permeabilities for ribose, arabinose, glucose, and sucrose that they report result from disruptions and loss of selectivity that occur in films of such high conductances (Cass et al., 1970).

reported by Finkelstein and Cass (1968) for nystatin-treated membranes arose from the same problem.

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