J. Membrane Biol. 41, 65–86 (1978)

Effects of Amphotericin B on the Electrical Properties of *Necturus* Gallbladder: Intracellular Microelectrode Studies

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Received 14 November 1977; revised 16 January 1978

Summary. Intracellular microelectrode techniques were employed to study the mechanism by which amphotericin B induces a transient mucosa-negative transepithelial potential (ΔV_{ms}) in the gallbladder of *Necturus*. When the tissue was incubated in standard Na-Ringer's solution, the antibiotic reduced the apical membrane potential by about 40 mV, and the basolateral membrane potential by about 35 mV, whereas the transepithelial potential increased by about 5 mV. The electrical resistance of the apical membrane fell by 83%, and that of the basolateral membrane by 40%; the paracellular resistance remained unchanged. Circuit analysis indicated that the equivalent electromotive forces of the apical and basolateral membranes fell by 35 and 11 mV, respectively. Changes in potentials and resistances produced by ionic substitutions in the mucosal bathing medium showed that amphotericin B produces a nonselective increase in apical membrane small monovalent cation conductance (K, Na, Li). In the presence of Na-Ringer's on the mucosal side, this resulted in a reduction of the K permselectivity of the membrane, and thus in a fall of its equivalent emf. During short term exposure to amphotericin B, P_{Na}/P_{Cl} across the paracellular pathway did not change significantly, whereas P_K/P_{Na} doubled. These results indicate that ΔV_{ms} is due to an increase of g_{Na} across the luminal membranes of the epithelial cells (Cremaschi *et al.*, 1977. *J. Membrane Biol.* **34**:55); the data do not support the alternative hypothesis (Rose & Nahrwold, 1976. *J. Membrane Biol.* **29**:1) that ΔV_{ms} results from a reduction in shunt P_{Na}/P_{Cl} acting in combination with a rheogenic basolateral Na pump.

Key words: Gallbladder, amphotericin B, leaky epithelia, sodium transport, membrane permeability.

Cremaschi and coworkers (1971) first showed that exposure of the luminal side of the rabbit gallbladder to the polyene antibiotic amphotericin B results in a mucosa-negative transmural potential of several mV. On the basis of transepithelial measurements, these authors concluded that the mechanism of this effect might be an increase of Na conductance at the luminal membrane of the epithelial cells, similar to the effect of amphotericin B on other salt-transporting epithelia (Bentley, 1968; Niel-

0022-2631/78/0041-0065 $02.40

sen, 1971; Candia, Bentley & Cook, 1974; Stroup *et al.*, 1974). Recently, Rose and Nahrwold (1976) have challenged this interpretation and proposed that the antibiotic reduces the cation selectivity of the limiting junctions of the epithelium and "unmasks" a rheogenic Na transport mechanism at the basolateral membranes of the cells. They speculated that in physiologic conditions this rheogenic pump (which tends to make the mucosal medium negative) is not evident because of (i) an opposing diffusion potential at the junctions: if active transport results in an increased [NaCl] in the lateral intercellular spaces, a mucosa-positive diffusion potential develops, because $P_{Na} > P_{Cl}$ across the junctions (Machen & Diamond, 1969); (ii) the large conductance of the intercellular pathway (Frömter, 1972; Hénin & Cremaschi, 1975; Reuss & Finn, 1975*a*; van Os & Slegers, 1975). The observations of Rose and Nahrwold do not exclude alternative explanations. In particular, their microelectrode studies were insufficient to rule out conclusively the hypothesis advanced by Cremaschi and coworkers.

The experiments reported here were designed to examine these two possibilities, with intracellular microelectrode techniques, in gallbladder epithelium of *Necturus maculosus*. This tissue is advantageous because the large cell size facilitates intracellular microelectrode studies. Our results do not provide evidence for rheogenic Na extrusion, and indicate that the main action of amphotericin B is to increase luminal membrane Na conductance. In addition, shortly after application of the antibiotic, a highly K-selective intercellular pathway develops, with no significant change in P_{Na}/P_{Cl}. During the preparation of this manuscript, Cremaschi *et al.* (1977) and Hénin *et al.* (1977) published results of similar experiments in gallbladders from rabbit and other species, reaching essentially the conclusions summarized above.

Materials and Methods

Necturi were obtained from Mogul-Ed Co., Oshkosh, Wisc., and kept in a large aquarium at 4 °C. The gallbladders were removed, mounted as previously described (Reuss & Finn, 1975*a*, 1977*a*), and incubated in Ringer's solution of the following composition (in mmoles per liter): NaCl, 109.2; KCl, 2.5; $CaCl_2$, 0.89; $NaHCO_3$, 2.38. The pH was about 8.0, after equilibration with room air. Ion replacements were isomolar. NaCl in the mucosal bathing medium was completely replaced with the chloride salts of K, Li, tetraethylammonium (TEA), or N-methyl-D-glucamine (NMDG). Dilution potentials (Barry, Diamond & Wright, 1971) were measured by exposure of the mucosal side to a solution containing half of the control NaCl concentration, with sucrose added to keep the osmolality constant. Both dilution and biionic potentials were corrected for the

respective liquid junction potentials measured in the cell Ag – AgCl/NaCl-Ringer's/agar-NaCl-Ringer's bridge/Solution X/Ag – AgCl, where solution X is standard NaCl-Ringer's or the substituting solution. The measured potential differences were corrected for the change of electrode potential caused by differences in Cl activity (according to the Guggenheim assumption) and by the presence of sucrose (Barry & Diamond, 1970). Amphotericin B was added to the mucosal bathing solution to a final concentration of 4.5 $\mu g \times ml^{-1}$ (4.9×10^{-6} M).

Electrical Measurements

Potentials and resistances were measured as previously described (Reuss & Finn, 1975*a*, *b*; 1977*a*). Glass microelectrodes were prepared from omega-dot tubing (Frederick Haer and Co., Brunswick, Maine) and filled with 3 M KCl or 4 M potassium acetate. Tip resistance ranged from about 15 to about 50 MΩ. Extracellular electrodes were Ag – AgCl pellets connected to the bathing solutions with agar-Ringer's bridges.

All tissues were tested after at least 30 min incubation in the chamber and accepted when their transepithelial resistance was larger than $200\,\Omega \times cm^2$, and when a 2:1 mucosal NaCl dilution resulted in a change of transepithelial potential of at least 8 mV. Cell impalements were performed with mechanic micromanipulators, during direct observation, at 300×, with a MS phase contrast inverted microscope (Nikon Inc., Garden City, N.Y.). Cell and/or transepithelial potentials were measured with high impedance ($> 10^{12}\,\Omega$) electrometers (provided with digital readouts to 0.1 mV) and displayed on a storage oscilloscope (Tektronix, Beaverton, Ore.) and on a dual channel pen recorder (Brush 2400, Gould Inc., St. Louis, Mo.). The reference electrode was always placed in the serosal solution.

The effects of amphotericin B were evaluated in two ways: (i) continuous microelectrode recording from a single cell, before, during, and after exposure to the antibiotic, and (ii) multiple impalements during the three periods.

The transepithelial resistance (R_t) was calculated from the transepithelial potential change (ΔV_{ms}) 50 msec after the start of a calibrated transepithelial current pulse of about $50\,\mu A \times cm^{-2}$. The ratio of resistances of the cell membranes (R_a/R_b, apical/basolateral) was computed from $\Delta V_{mc}/\Delta V_{cs}$ upon passage of transepithelial current.

Calculation of Resistances and Equivalent Electromotive Forces at the Cell Membranes and the Shunt Pathway

According to the equivalent circuit shown in Fig. 1, the values of R_a, R_b, and R_s can be computed from R_t, R_a/R_b, and $R_z = R_a \cdot R_b/(R_a + R_b)$, i.e., the specific resistance for current flow from the cells into the bathing media. R_z can be determined from cable analysis as described by Frömter (1972). Because R_z changes continuously after exposure to amphotericin B, short-time changes in R_a, R_b, and R_s were computed from cable analyses performed in the following way: two cells were impaled, and intracellular current pulses (2×10^{-8} A) were passed through one microelectrode at 10- to 20-sec intervals. The steady-state potential change in the second cell (V_x) was recorded. The interelectrode distance (x) was measured with a calibrated micrometer eyepiece. The mucosal medium was changed to one containing amphotericin B and the cell potentials and V_x values were recorded for several min (up to 5). When approximately steady-state changes of V_{ms}, V_{cs}, and V_x were reached, the antibiotic was washed off. Only experiments in which V_{cs} returned to within 1 mV and V_x to within 10% of control were included. Two sets of V_x values (control and experimental) were obtained from three impalements in

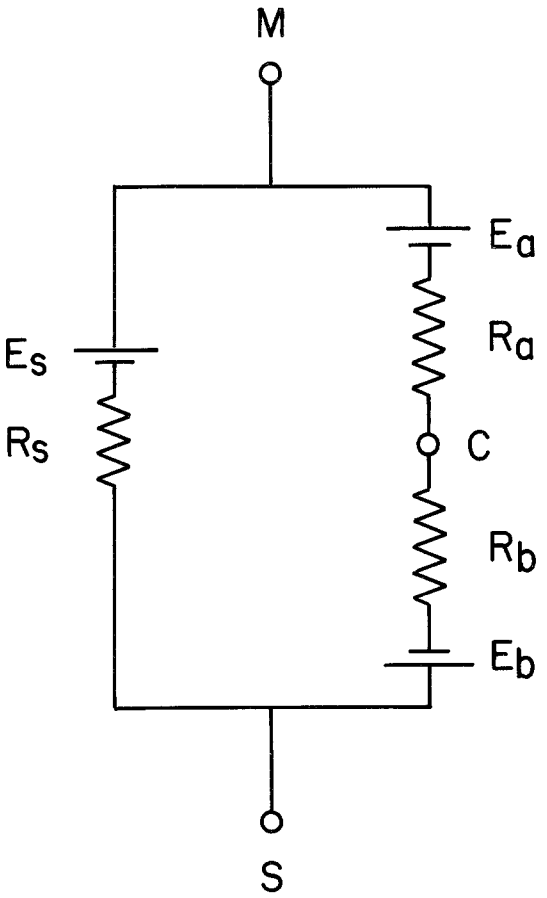

Fig. 1. Equivalent electrical circuit for *Necturus* gallbladder epithelium. *M*, *C*, and *S* represent the mucosal solution, the cell, and the serosal solution, respectively. Each element of the circuit is represented by a Thevenin equivalent (equivalent emf (*E*'s) in series with an equivalent resistance (*R*'s)). The subscripts *a*, *b*, and *s* refer to the apical membrane, basolateral membrane, and shunt pathway, respectively

each of four preparations, plotted as function of x and fitted by the Bessel function K_0. From the best fits, the values of R_z in the two conditions were calculated as described before (Reuss & Finn, 1975*a*). A substantial dispersion was observed, from tissue to tissue, in particular at $x > 200$ μm. However, in every case V_x decreased sizeably during exposure to amphotericin B. The fractional decrease of V_x ($\Delta V_x/V_x$) increased with the interelectrode distance, indicating a reduction of the space constant for intracellular current spread. When the control values of V_x were normalized (by addition or subtraction) to the best fit curve, and the experimental values of V_x were corrected exactly as the respective controls, the dispersion of the points obtained in amphotericin became minimal. This shows that the dispersion of the crude data is due to tissue to tissue variation, which can result from real differences in the specific resistances of the cell membranes and/or different degrees of stretch of the preparations.

R_t and R_a/R_b were measured in these and other preparations, before and during exposure to amphotericin B. Control and experimental values of R_a, R_b and R_s were calculated from R_t, R_a/R_b, and R_z in each condition (Reuss & Finn, 1975*a*).

The equivalent electromotive forces of the cell membranes and the shunt pathway (E_a, E_b, and E_s, respectively, *see* Fig. 1) were computed from the resistances and the measured values of the transepithelial potential (V_{ms}), and the cell membrane potentials (apical: V_{mc}, basolateral: V_{cs}). E_s was assumed to be zero with symmetric bathing solutions (Reuss & Finn, 1975*a*, *b*). Other assumptions, and their justification, are detailed below.

Calculation of Shunt Ionic Permeability Ratios

Paracellular permeability ratios were calculated from the changes of potentials and resistances measured during fast changes in the composition of the mucosal bathing solution. A microelectrode was kept in a cell during and after the substitution. If one assumes that over a brief period R_b and V_b remain constant, the changes of E_a and E_s (ΔE_a, ΔE_s) can be computed as described before (Reuss & Finn, 1975*b*). P_{Cl}/P_{Na} and P_K/P_{Na} were calculated from the solution of the constant field equation written for Na, Cl, and K, for the E_s changes resulting from mucosal NaCl dilution and K-for-Na replacement. Activities were used instead of concentrations.

Statistics

The results are expressed as means $\pm$SEM. Comparisons were made by conventional paired data analysis.

Results

Effect of Amphotericin B on Transepithelial Potential and Resistance

As observed by others in gallbladders of other species, the addition of amphotericin B to the mucosal solution bathing *Necturus* gallbladder produces a mucosa-negative change of V_{ms} and a small, but highly significant, decrease of R_t. These changes are summarized in Table 1. A

Table 1. Effects of amphotericin B on transepithelial potential (V_{ms}) and transepithelial resistance (R_t)

	V_{ms} (mV)	R_t (Ω cm^2)
Control	-1.5 ± 0.3	340 ± 25
Amphotericin B	-6.6 ± 1.3	315 ± 25
Δ	5.1 ± 1.1	25 ± 4
P	<0.005	<0.001

Amphotericin B values of V_{ms} and R_t measured at the peak action of the antibiotic on V_{ms} (*see* Fig. 2). Reference: serosal solution. $N = 10$ experiments.

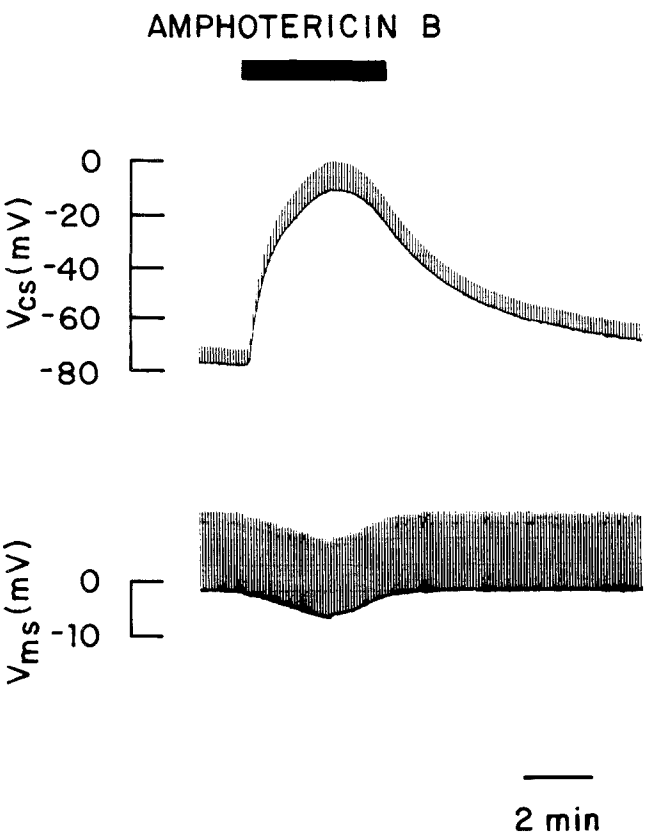

Fig. 2. Typical record of potentials and resistances before, during, and after addition of amphotericin B to the mucosal medium of a preparation bathed with Na-Ringer's on both sides. *Upper panel:* basolateral membrane potential (V_{cs}). *Lower panel:* transepithelial potential (V_{ms}). Upper record starts with the microelectrode in a cell. Exposure of the tissue to amphotericin B (4.5 μg/ml, mucosal side) indicated by the bar. Note that V_{ms} increased (by about 5 mV) and V_{cs} decreased (by about 68 mV). V_{mc}, not shown, fell by 73 mV ($\Delta V_{mc} = \Delta V_{cs} + \Delta V_{ms}$). The upward deflections in both records were caused by transepithelial current pulses of constant density throughout the experiment. The increase in the V_{cs} deflection (while the V_{ms} deflection does not change), during exposure to the antibiotic, indicates that R_a/R_b falls: $(\Delta V_{ms} - \Delta V_{cs})/\Delta V_{cs} = \Delta V_{mc}/\Delta V_{cs} = R_a/R_b$. R_t did not change (as shown in this record) or decreased slightly during exposure to amphotericin B. Finally, this experiment illustrates that if exposure to the drug is brief, its effects on potentials and resistances are reversible

typical record is shown in the bottom tracing of Fig. 2. At this concentration, the effects of amphotericin B are reversible, if exposure is reduced to less than ca. 10 min. Prolonged action of the antibiotic results in a continuous, irreversible drop of R_t and V_{ms}.

Table 2. Effects of amphotericin B on cell membrane potentials

	Apical, V_{mc} (mV)	Basolateral, V_{cs} (mV)
Control	-68.1 ± 4.5	-69.6 ± 4.8
Amphotericin B	-28.0 ± 3.9	-34.6 ± 4.0
Difference	-40.1 ± 4.5	-35.0 ± 4.3
P	<0.001	<0.001

Values in amphotericin B measured at the peak action of the antibiotic on V_{ms}. Reference: respective bathing medium. $N=10$ (same experiments as in Table 1).

Effects of Amphotericin B on Cell Membrane Potentials and Resistances

A typical record of the change of V_{cs} after exposure to amphotericin B is shown in the upper tracing of Fig. 2. V_{mc}, not shown in the record, fell more than V_{cs} (since V_{ms} became more negative during the action of the drug). Values of V_{mc} and V_{cs} in several preparations, under control conditions and at the maximum effect of amphotericin B, are shown in Table 2. V_{mc} and V_{cs} always decreased. Cell potential changes were far larger than the transepithelial potential changes summarized in Table 1 ($\Delta V_{mc}/\Delta V_{ms}=8$, $\Delta V_{cs}/\Delta V_{ms}=7$). As illustrated in Fig. 2, the changes in potentials brought about by amphotericin B were slowly reversible, at this dose ($4.5\ \mu g \times ml^{-1}$), provided that the time of exposure was less than ca. 10 min. Virtually identical results were obtained when cell potentials were measured in several cells under control conditions and after the full effect of the antibiotic.

In four experiments in which all chloride was replaced with sulfate (adding sucrose to isomolality) or isethionate, the effects of amphotericin B on transepithelial and cell membrane potentials were similar to those observed in NaCl-Ringer: $\Delta V_{ms}=4.9 \pm 0.8$, $\Delta V_{mc}=37.0 \pm 4.2$, $\Delta V_{cs}=32.1 \pm 3.9\ mV$ (compare with Tables 1 and 2). In most of the experiments reported here the addition of amphotericin B to the mucosal medium resulted in a decrease of the transepithelial resistance (R_t). Although small, this change was highly significant (*see* Table 1). As exemplified in Fig. 2, the ratio of cell membrane resistances (R_a/R_b) fell dramatically under the action of the antibiotic: from 1.29 ± 0.11 to 0.36 ± 0.07 (difference $=0.93 \pm 0.13$, $p<0.001$). These results are consistent with a decrease of R_a. However, R_a/R_b could fall because R_b increases, and a concomitant drop of R_s could account for the reduction of R_t. To distinguish between

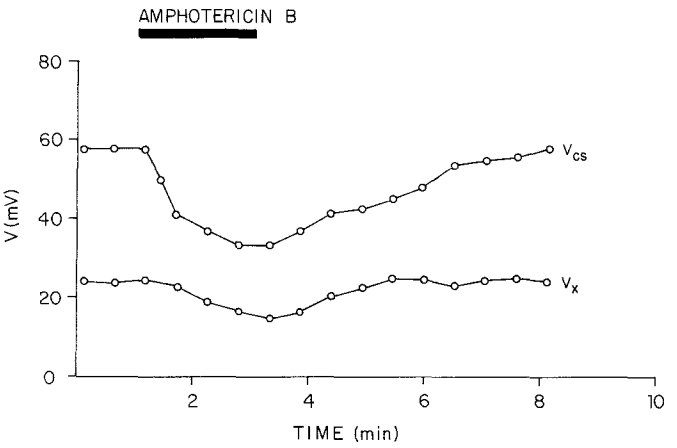

Fig. 3. Typical result of cable analysis before, during, and after exposure to amphotericin B on the mucosal side. Two microelectrodes were kept in cells about 35 μm apart. Intracellular negative pulses of 2×10^{-8} A were applied through one of the microelectrodes at about 15-sec intervals. The second microelectrode was used to record the basolateral membrane potential (V_{cs}) and the intracellular voltage deflections (V_x) elicited by the intracellular current pulses. Note that V_x falls reversibly during exposure to amphotericin B, concomitantly with the change of V_{cs}. From V_x values under control conditions and during exposure to amphotericin B, R_z was computed for each condition, as explained in the text

these two possibilities, the experiment illustrated in Fig. 3 was performed. As described above, the basolateral membrane potential (V_{cs}), and the voltage change elicited in the same cell by passing current through another cell (V_x) were monitored continually before, during, and after exposure to the drug. As observed in this and other similar experiments, both V_{cs} and V_x fell reversibly. This observation proves that amphotericin B reduces R_z: V_x is a direct function of R_z, i.e., the resistance for current flow out of the cells ($R_z = R_a \cdot R_b/(R_a + R_b)$). As shown before, R_a/R_b fell as a consequence of the action of the antibiotic. Both results can be explained only by a reduction of R_a (regardless of any change of R_b).

Cell membrane and shunt resistances were estimated in several tissues from the values of R_t and R_a/R_b before and after amphotericin B, and from R_z in the two conditions, calculated, as described above, from cable analysis in a different set of gallbladders. The cell membrane resistance results, shown in Table 3, indicate that amphotericin B produces a large decrease of R_a, and a smaller reduction of R_b.

The equivalent electromotive forces of the cell membranes were calculated from the mean values of potentials and resistances before and

Table 3. Effects of amphotericin B on cell membrane resistances

	R_a (Ω cm^2)	R_b (Ω cm^2)
Control	4440 ± 100	3440 ± 120
Amphotericin B	740 ± 20	2050 ± 230
Difference	3700 ± 100	1390 ± 240
P	<0.001	<0.001

$N = 10$ experiments. Resistances computed from R_t, $a = R_a/R_b$, and R_z, as described in the text.

Table 4. Effects of amphotericin B on E_a and E_b

	E_a (mV)	E_b (mV)
Control	−49.3	−84.1
Amphotericin B	−14.2	−72.7

Negative polarities indicate cell negative to the respective bathing medium. Values calculated from means in Tables 1–3, and 7, according to Eqs. (1) and (2). (*See text.*)

during the action of amphotericin B (Tables 1–3). Assuming that $E_s = 0$ in the two conditions,

$$E_a = V_{mc} - V_{ms} \frac{R_a}{R_s}, \tag{1}$$

$$E_b = V_{cs} + V_{ms} \frac{R_b}{R_s}. \tag{2}$$

The results, shown in Table 4, indicate a large decrease of E_a and a moderate reduction of E_b. Since the control and experimental values of E_s are uncertain, E_a and E_b were also calculated for E_s values of up to 2 mV (mucosa positive), according to the standing osmotic gradient hypothesis and the Na/Cl selectivity of the shunt pathway (Machen & Diamond, 1969). If E_s remains unchanged by the antibiotic, both E_a and E_b drop regardless of the assumed value of E_s. If E_s falls in amphotericin B (as proposed by Rose & Nahrwold), the amphotericin B-dependent fall of E_a is smaller and the fall of E_b larger than shown in Table 4. No reasonable combination yields an increase of E_b.

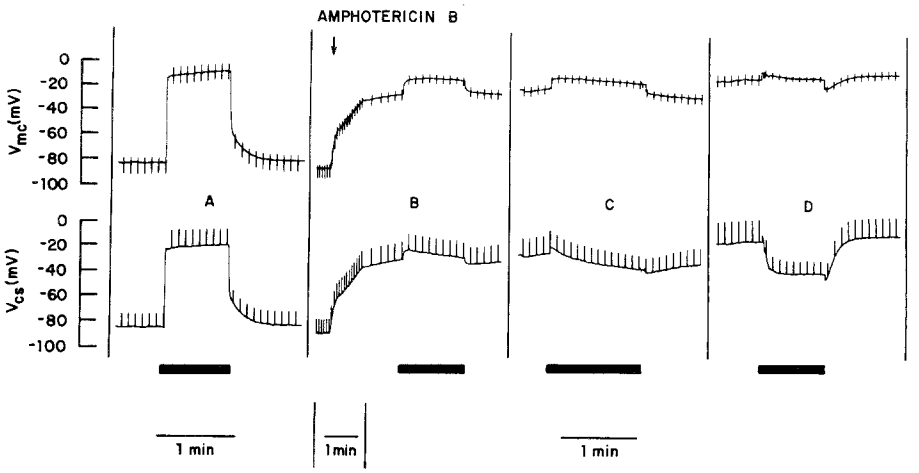

Fig. 4. Effects of amphotericin B on potential and resistance changes induced by K – Na substitutions on the mucosal side. *Upper record:* apical membrane potential (V_{mc}); *lower record:* basolateral membrane potential (V_{cs}). Polarity convention: V_{cell} referred to the respective bathing medium. Amphotericin B was present continuously after addition in panel *B* (arrow). Panel *A*: control. Tissue exposed to K-Ringer's during the time indicated by bar. Note large depolarization of both membranes and reduction of the ratio of potential deflections induced by transepithelial current. Panel *B*: initial slow record, effect of amphotericin on V_{mc}, V_{cs} and R_a/R_b (*compare* Fig. 2). During exposure to K-Ringer's, the magnitude of the instantaneous changes of V_{mc} and V_{cs} is reduced to about 18% and 13% of the respective control values. Panels *C* and *D*: K-induced instantaneous depolarization is reduced further. A late hyperpolarizing phase is evident during exposure to K. $\Delta V_{cs} \gg \Delta V_{mc}$, indicating a large increase of ΔV_{ms} during exposure to K-Ringer's. The intervals between panels were 2, 0.5, and 3 min, respectively. For further details, *see text*

Effects of Amphotericin B on Apical Membrane Cationic Selectivity

The simplest explanation for the reduction of E_a effected by the antibiotic is a decrease of apical membrane K permselectivity. To test this hypothesis, potentials and resistances were measured before and during brief mucosal solution substitutions of Na for K, both under control conditions and in the presence of amphotericin B.

The result of a typical experiment is illustrated in Fig. 4. Under control conditions, exposure to K-Ringer's on the mucosal side resulted in immediate large drops of V_{mc} and V_{cs} (ΔV_{mc} and ΔV_{cs}). Since $\Delta V_{mc} > \Delta V_{cs}$, V_{ms} became more negative immediately after the change in bathing solution. R_a/R_b underwent a large decrease, and R_t fell slightly. During the period of exposure to K-Ringer's, V_{mc} and V_{cs} depolarized further (as shown in the figure), remained constant, or, more frequently,

hyperpolarized slightly. V_{ms}, not shown, constantly hyperpolarized. After exposure to amphotericin B both the immediate and late ΔV's induced by the K-for-Na substitution were altered:

1) The immediate changes of V_{mc} and V_{cs} were considerably reduced as compared to control, but took place in the same direction: both membranes depolarized by exposure to K-Ringer's, before and after addition of the antibiotic.

2) The late cell membrane potential changes were in the hyperpolarizing direction in the presence of amphotericin B; the V_{cs} change was always far larger than the V_{mc} change, but both values were significantly higher than the respective controls. The slow change of V_{ms}, not shown in the figure, followed closely the V_{cs} change. These late hyperpolarizing voltage transients became noticeable a few minutes after addition of amphotericin B, increased to reach a maximum after 5 to 10 min, and decreased progressively thereafter, concomitantly with a large, irreversible drop of R_t and tissue ionic selectivity.

Resistance changes produced by exposure to K-Ringer's on the mucosal side were also altered by the action of amphotericin B: R_a/R_b, already diminished in Na-Ringer's + amphotericin B, fell less than in the absence of the antibiotic, whereas R_t decreased (as a consequence of the K-for-Na substitution) more in the presence than in the absence of amphotericin B. Note that the voltage deflection produced across the apical membrane by the transepithelial current pulses is biphasic. This is due to an unstirred layer transport number effect, which results in depolarization of both cell membranes during pulses in the mucosa to serosa direction (Reuss & Finn, 1977*b*). R_a/R_b was calculated from the instantaneous changes of V_{mc} (downwards) and V_{cs} (upwards), recorded on a faster time base.

Mean immediate changes of potentials and resistances produced by exposure to K-Ringer's on the mucosal side, in the presence and absence of amphotericin B, are shown in Table 5. Note that the depolarization of both cell membranes produced by exposure to K-Ringer's was largely reduced by amphotericin B. This result is consistent with the hypothesis of a drop of apical membrane K permselectivity. R_a decreased by exposure to K-Ringer's both under control conditions and in the presence of the antibiotic. However, both the absolute and fractional reduction of R_a were smaller during exposure to amphotericin B.

Applying the assumptions described below, the equivalent electromotive forces of the cell membranes and the shunt pathway were calculated from the mean values shown in Table 5. The assumptions

Table 5. Effects of mucosal exposure to K-Ringer's on potentials and resistances

Condition	Bathing medium	V_{ms}	V_{mc}	V_{cs}	R_t	R_a/R_b	R_a	R_b	R_s
Control	Na-Ringer's	-1.7 ± 0.1	-70.6 ± 3.8	-72.3 ± 3.8	284 ± 6	1.32 ± 0.14	3480	2636	298
	K-Ringer's	-12.7 ± 0.9	-6.4 ± 2.4	-19.1 ± 2.4	243 ± 6	0.40 ± 0.08	1054	2636	260
	Difference (Na − K)	11.0 ± 0.9	-64.2 ± 2.7	-53.2 ± 2.7	41 ± 7	0.92 ± 0.10			
Amphotericin B	Na-Ringer's	-5.2 ± 0.4	-12.6 ± 2.9	-17.8 ± 3.3	249 ± 8	0.26 ± 0.03	504	1938	281
	K-Ringer's	-12.8 ± 1.1	-0.2 ± 2.1	-13.0 ± 2.9	219 ± 9	0.21 ± 0.01	407	1938	245
	Difference (Na − K)	7.6 ± 0.6	-12.4 ± 1.3	-4.8 ± 0.8	30 ± 3	0.05 ± 0.02			

Potentials, mV; resistances, $\Omega\,cm^2$; $n=7$ experiments. K-Ringer's values correspond to measurements obtained immediately after the mucosal solution substitution. Resistances calculated from R_z and means of R_t and R_a/R_b, assuming that R_b remains constant shortly after the K-for-Na substitution. All differences are significant, with $P<0.005$ or better, except R_a/R_b in amphotericin B (nonsignificant)

Table 6. Estimated values of cell membrane and shunt equivalent electromotive forces in tissues bathed in Na- or K-Ringer's, in the presence or absence of amphotericin B

Condition	Bathing medium	E_a	E_b	E_s
Control	Na-Ringer's	−50.8	−87.3	0
	K-Ringer's	+20.9	−87.3	−6.0
	Difference (Na − K)	−71.7	–	6.0
Amphotericin B	Na-Ringer's	−3.3	−53.7	0
	K-Ringer's	+8.4	−53.7	−7.7
	Difference (Na − K)	−11.7	–	7.7

EMF's in mV, calculated from means shown in Table 5.

were: (i) $E_s=0$ when the tissue is exposed to Na-Ringer's on both sides, in the presence or absence of the antibiotic; (ii) R_b and E_b do not change shortly after the K-for-Na mucosal solution substitution. Solution of the appropriate circuit analysis equations (Reuss & Finn, 1975*a–b*) yielded the results shown in Table 6. The prominent feature is the large drop of the K-dependent E_a change during amphotericin B. T_K (i.e., the K-dependent partial emf ratio) across the luminal membrane was estimated from the equation

$$T_K=\frac{\Delta E_a}{\frac{RT}{zF}\ln\frac{C_1}{C_2}} \tag{3}$$

where ΔE_a is the K-Ringer's-induced change of apical membrane emf and C_1 and C_2 are the K concentrations in K-Ringer's and Na-Ringer's, respectively. T_K was 0.74 under control conditions and 0.12 in amphotericin B, indicating a drop of apical membrane K permeability relative to that for other ions. Since similar effects of amphotericin B on potentials and resistances are observed in tissues incubated in Cl-free media, one must conclude that the reduction of apical membrane T_K caused by the antibiotic is due to an increase of sodium conductance.

The changes of potentials, resistances, and emf's observed during prolonged exposure to K-Ringer's are more difficult to interpret because of the likely possibility of ion concentration changes in the cytoplasm of the epithelial cells (*see Discussion*).

Effects of Amphotericin B on Paracellular Resistance and Ionic Selectivity

As shown in Table 1, amphotericin B produced a small, but significant, decrease of transepithelial resistance (R_t). A similar effect was observed in the series of experiments shown in Table 5. This reduction appeared to be the consequence of the drop of transcellular resistance induced by the antibiotic (*see* Table 3). The shunt resistance remained unchanged, as shown in Table 7.

Table 7. Effects of amphotericin B on paracellular resistance and selectivity

	R_s (Ω cm^2)	ΔV_{ms} (dilution) (mV)	ΔV_{ms} (biionic K−Na) (mV)	
			Immediate	Steady state
Control	355±20	12.4±0.6	−11.0±0.9	−13.6±2.5
Amphotericin B	355±30	11.7±0.6	−7.6±0.6	−26.3±2.5
Difference	0±10	0.7±0.4	−3.4±0.8	12.7±3.4
P	NS	NS	<0.01	<0.01

R_s: shunt resistance, calculated from the individual data of 10 preparations (Tables 1 and 3). ΔV_{ms} (dilution): V_{ms} change produced by isosmotic replacement of half of the NaCl in the mucosal medium ($n=8$). ΔV_{ms} (biionic K−Na): V_{ms} change produced by complete mucosal replacement of Na with K ($n=7$). Immediate change measured at the peak effect of the ionic substitution on cell potentials. Steady-state change measured when V_{ms} became stable.

The selectivity of the shunt pathway was studied by the measurement of transepithelial NaCl dilution and Na/K biionic potentials. Under control conditions, ΔV_{ms} (i.e., the change of transepithelial potential) and ΔE_s (i.e., the change of shunt emf) are very similar because $R_s \ll (R_a + R_b)$ (*see* Reuss & Finn, 1975*b*). In amphotericin B, however, the reduction of transcellular resistance results in a significant contribution of the cell membranes to ΔV_{ms}. For this reason, ΔE_s had to be estimated to calculate ion permeability ratios (*see Discussion*). The ΔV_{ms} data is shown in Table 7. The presence of amphotericin B did not alter the value of ΔV_{ms} caused by a 2:1 NaCl dilution on the mucosal side, provided that the measurement was made within 10 min of the addition of the antibiotic. Later on, ΔV_{ms} fell significantly. K-Na substitutions resulted in a smaller immediate change of V_{ms} in the presence of amphotericin B, as compared to the control condition. The steady-state ΔV_{ms} value, however, was significantly higher in amphotericin B.

Effect of Amphotericin B in the Absence of Na in the Mucosal Medium

The experiments described above suggest that amphotericin B opens an apical membrane pathway permeable to K and Na. Additional information on the properties of this path was obtained from experiments in which all mucosal Na was replaced with other cations of different radii: lithium, *n*-methyl-D-glucamine (NMDG) and tetraethylammonium (TEA).

A typical Li experiment is illustrated in Fig. 5. Amphotericin B produced large decreases of V_{cs} and V_{mc} (not shown), whereas V_{ms} became negative (the positive value in Li-Ringer's before amphotericin B was caused by a shunt biionic potential: $P_{Na} > P_{Li}$). The effect of the antibiotic was slowly and only partially reversible. The two-phase depolarization of the cell membrane shown in the figure was a constant feature. Its explanation is uncertain. The results of six such experiments are summarized in Table 8. In Li-Ringer's, although the changes are smaller, the results were qualitatively similar to those in Na-Ringer's.

As shown also in Table 8, when larger cations (NMDG or TEA) replaced Na, amphotericin B did not significantly alter the transepithelial or the cell potentials.

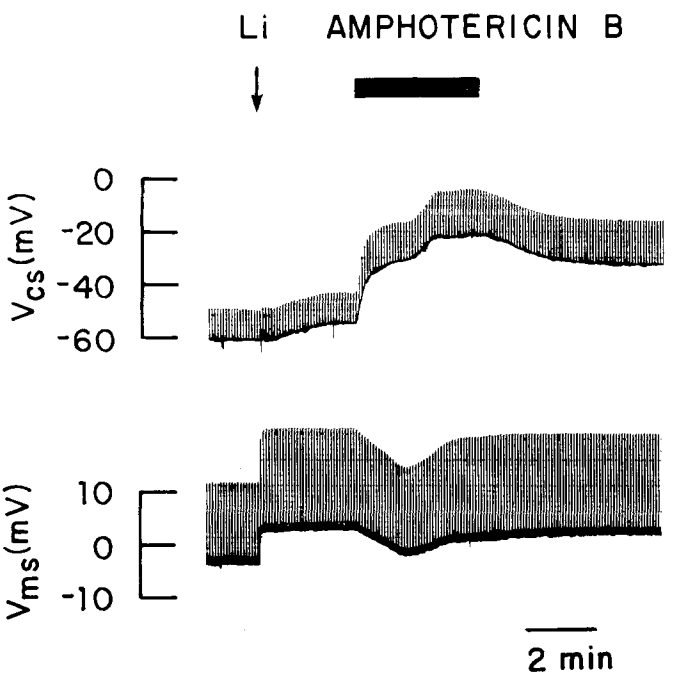

Fig. 5. Effect of amphotericin B on potentials and resistances during exposure to Li-Ringer's on the mucosal side. Protocol as in Fig. 2. At the arrow, Na in the mucosal medium was completely replaced with Li. The positively oriented changes of V_{ms} and V_{cs} are the result of a shunt biionic potential ($P_{Na} > P_{Li}$). Note also the increase of R_t (proportional to the V_{ms} deflections). Exposure to amphotericin B is indicated by the bar. It results in a negatively oriented change of V_{ms} and a drop of V_{cs}. The depolarization of V_{mc}, not shown, is larger than that of V_{cs} ($\Delta V_{mc} = \Delta V_{cs} + \Delta V_{ms}$). Resistance changes are comparable to those observed in Na-Ringer's. Reversibility is slower, and usually only partial. The biphasic course of V_{cs} during exposure to amphotericin B was a constant feature

Table 8. Changes of transepithelial and cell potentials produced by amphotericin B in the absence of Na in the mucosal medium

	ΔV_{ms} (mV)	ΔV_{mc} (mV)	ΔV_{cs} (mV)
Li-Ringer's	4.2 ± 0.8	-34.6 ± 3.9	-30.5 ± 3.3
P	<0.005	<0.001	<0.001
X-Ringer's	1.1 ± 0.9	6.6 ± 6.5	5.6 ± 5.6
P	NS	NS	NS

Li-Ringer's. $n=6$. X-Ringer's = Na replaced with TEA or NMDG. $n=6$. Values are the peak changes of potentials caused by amphotericin B.

Discussion

As observed previously in gallbladders of several species (Cremaschi *et al.*, 1971; Rose & Nahrwold, 1976; Cremaschi *et al.*, 1977; Hénin *et al.*, 1977), in *Necturus* gallbladder amphotericin B produces a rather large change of transepithelial potential, in the mucosa negative direction.

Inspection of the equivalent circuit shown in Fig. 1 indicates that the observed increase of V_{ms} can in theory result from a variety of changes of emf's and/or resistances. These possibilities can be easily considered from the following equation:

$$V_{ms} = \frac{(E_b - E_a) R_s - E_s(R_a + R_b)}{R_a + R_b + R_s} \tag{4}$$

where positive values indicate: V_{ms}, mucosa negative; E_s, mucosa positive; E_b and E_a, cell negative.

The observed mucosa-negative change of V_{ms} could result from one or more of the following mechanisms:

1) A decrease of E_s, which under control conditions would be mucosa positive because of a higher NaCl concentration in the lateral intercellular spaces than in the mucosal medium, and because $P_{Na} > P_{Cl}$ across the limiting junctions (Machen & Diamond, 1969). This mechanism has been proposed by Rose and Nahrwold (1976) to account in part for the effect of amphotericin B on V_{ms} in rabbit gallbladder.

2) A decrease of E_a, which under control conditions is mainly dependent on the K concentration gradient across the luminal membrane (Hénin & Cremaschi, 1975; Reuss & Finn, 1975*a–b*; van Os & Slegers, 1975). This mechanism, i.e., a reduction of apical membrane K permselectivity, has been proposed by Cremaschi *et al.* (1977) as the explanation for the effect of amphotericin B on V_{ms}.

3) An increase of E_b. For instance, if the Na pump located in the basolateral membrane is electrogenic (i.e., if ion flow through the pump is not neutral, but results in net transfer of charge), increased Na entry from the mucosal medium could stimulate the pump, increase the pump current and E_b, and hyperpolarize V_{ms}. This mechanism was also proposed by Rose and Nahrwold.

4) An increase of R_s, because of the larger voltage drop through the shunt of the circular current generated by $(E_b - E_a)$. The data presented here and the results of Cremaschi *et al.* (1977) do not support this possibility, because R_s does not rise.

5) A decrease of (R_a+R_b), which can result in a larger V_{ms} by two mechanisms: first, an increase of the ratio $R_s/(R_a+R_b+R_s)$, which would result in a larger effect of (E_b-E_a) on V_{ms}, and second, a decrease of the ratio $(R_a+R_b)/(R_a+R_b+R_s)$, which would minimize the effect of E_s on V_{ms}.

The experiments reported here were designed to identify which of these mechanisms is (are) responsible for the observation of an increased value of V_{ms} in the presence of amphotericin B. The results indicate that amphotericin B increases the conductance of the luminal membrane of the epithelial cells to small cations, reducing its K selectivity, and therefore causing a fall of E_a. In addition, the large fall of transcellular resistance, in the absence of sizeable changes of R_s, contributes to the amphotericin B-dependent transepithelial hyperpolarization, by the mechanism described above (5). There is no evidence for a rheogenic mechanism of Na extrusion or for a reduction of P_{Na}/P_{Cl} across the shunt shortly after the action of the antibiotic.

In the next paragraphs, the evidence for the effects of amphotericin B at each element of the circuit, and the possible mechanisms involved, will be briefly analyzed.

Effects of Amphotericin B on the Luminal Membrane

The antibiotic causes: (i) increase of total membrane conductance (R_a falls), (ii) depolarization (caused mainly by a drop of E_a), and (iii) reduction of the dependence of E_a on external K concentration (i.e., a drop of T_K). The effects are similar in the absence of Cl. Therefore, with NaCl-Ringer's bathing the mucosal side the main apical action of the antibiotic is to increase g_{Na}. Since, in the presence of amphotericin B, E_a drops and R_a/R_b falls when the mucosal K concentration is increased, g_K remains larger than g_{Na}, but the fractional increase of g_{Na} is larger than that of g_K. The experiments in Li-Ringer's suggest that g_{Li} is raised as well, but less than g_{Na}. TEA and NMDG appear to be essentially impermeant both in the absence and presence of amphotericin B. In conclusion, the antibiotic "opens" a cation selective pathway across the luminal membrane, with the sequence $g_K > g_{Na} > g_{Li} \gg g_{TEA}, g_{NMDG}$.

Effects of Amphotericin B on the Basolateral Membrane

A reduction of R_b and a drop of E_b were computed even shortly after addition of the antibiotic to the mucosal solution. These changes are

smaller than those at the luminal membrane and subject to the potential errors involved by the assumptions necessary for the computation and the method employed for the cable analysis. They are, however, statistically significant. As discussed in analogous experiments in mammalian urinary bladder (Lewis *et al.*, 1977) effects of polyene antibiotics on the contralateral membrane of epithelial cells do not necessarily indicate penetration of the drug, but can result from changes of cell volume and/or ionic composition.

The possibility of an action of amphotericin B initially restricted to the luminal border was investigated in experiments in which the concentration or the duration of the exposure to the antibiotic were reduced. In these experiments, cell membrane and shunt resistances were calculated from R_t and R_a/R_b before and during the action of amphotericin B, assuming that only R_a is altered by the drug. The appropriate equations have been published elsewhere (Reuss & Finn, 1974). In six such experiments, it was impossible to obtain reliable estimates of the cell membrane resistances. The main reason appears to be that if only R_a changes, by amphotericin B, the effect on R_a/R_b is sizeable, but the effect on R_t is very small, because the latter is so close to R_s. An overestimation of R_t (in amphotericin B) of only 1% (ca. $3\,\Omega\,cm^2$) can result in a 50% overestimation of (R_a+R_b). This fact, and the evidence which indicates a rapid reduction of R_b, can explain the extremely low values of cell membrane resistances computed by Hénin *et al.* (1977) in gallbladders of several species, under the assumption of an effect of amphotericin B restricted to the luminal membrane.

Effects of Amphotericin B on the Shunt Pathway

The effects of the antibiotic on resistance and selectivity of the paracellular pathway are less important than those at the luminal membrane of the epithelial cells. R_s does not change significantly shortly after exposure to the drug. The estimation of P_{Na}/P_{Cl} (from the change of V_{ms} caused by a 50% reduction of mucosal NaCl concentration) reveals no change. In contrast, the magnitude of the V_{ms} change produced by exposure to K-Ringer's on the mucosal side [ΔV_{ms}(K)] changes significantly in the presence of amphotericin B, both immediately (i.e., at the peak of the effect on cell potentials) and after a steady state is reached. The immediate ΔV_{ms}(K) is significantly smaller in amphotericin B than

under control conditions. This result can be explained by the different changes of E_a obtained in the two conditions: a reduction of E_a tends to make the mucosal medium negative [*see* Fig. 1 and Eq. (4)]; since E_a drops less in amphotericin B, the effect on V_{ms} is also reduced. A calculation from the data of Table 5 indicates that this mechanism could reduce ΔV_{ms} by 4.8 mV, as compared to the observed reduction of 3.4 mV (*see* Table 7). At the steady-state, ΔV_{ms}(K) is roughly twice the immediate value. As explained below, most of this effect is due to a high shunt P_K/P_{Na}. An increase of E_a brought about by K entry across the luminal membrane after the immediate depolarization would tend to decrease V_{ms}. Therefore, the change of transepithelial potential has to result from an increase of E_b (cell negative) or an increase of E_s (mucosa negative) or a combination of both. If E_b increases, V_{ms}, V_{mc} and V_{cs} will increase, as it was observed experimentally. If only E_s increases, V_{ms} and V_{cs} would increase, whereas V_{mc} would decrease (*see* Fig. 1). The situation is too complicated to be analyzed quantitatively. However, assuming that at the steady state the K concentration in the cells is equal to that in the mucosal solution, and that $g_K = g_t$ across the basolateral membrane, E_b would be at most 95.1 mV, and ΔE_b 41.4 mV. Such a change of E_b would result in a V_{ms} hyperpolarization of only 3.9 mV. The rest (14.8 mV) is caused by the K/Na shunt biionic potential. From this value and the resistances, one can calculate that the minimum change of E_s during exposure to K-Ringer's in amphotericin is 16.4 mV.

To estimate P_{Cl}/P_{Na} and P_K/P_{Na} across the shunt, the constant field equation was solved simultaneously for the changes of shunt emf (ΔE_s) caused by mucosal NaCl dilution and K−Na replacement. Activities were used instead of concentrations. For the biionic potentials, ΔE_s values were employed. For the dilution potentials, ΔV_{ms} was assumed to be equal to ΔE_s. The error introduced by this simplification is small and results in an overestimation of P_{Cl}/P_{Na} and an underestimation of P_K/P_{Na}. Because of the direction of the changes, the assumption does not alter the conclusions. As shown in Table 9, P_{Cl}/P_{Na} does not change shortly after exposure to amphotericin B, whereas P_K/P_{Na} increases. The different P_K/P_{Na} values obtained from immediate and steady-state ΔV_{ms} means would support the notion of a faster change of E_a than of E_s upon K-for-Na replacement on the mucosal side, and therefore the possibility that the K selective barrier in the shunt is located deeply in the limiting junction. The reduction of R_t at the end of the period of exposure to K-Ringer's was larger in the presence than in the absence of amphotericin B (40 ± 10 and $120 \pm 5\ \Omega\,cm^2$, respectively). This observation is consistent

Table 9. Estimated shunt permeability ratios under control conditions and during exposure to amphotericin B

		From immediate changes	From steady-state changes
Control	P_{Cl}/P_{Na}	0.13	0.13
	P_K/P_{Na}	1.33	1.49
Amphotericin B	P_{Cl}/P_{Na}	0.16	0.14
	P_K/P_{Na}	1.45	2.97

P ratios calculated from data in Tables 5, 6, and 7, as explained in the text.

with the idea of the "opening" of a paracellular K-selective pathway by exposure to the antibiotic.

In sum, amphotericin B appears to have no effects on shunt P_{Cl}/P_{Na}, and to establish a K-selective paracellular pathway at the time of its full effect on V_{ms} and cell potentials. Prolonged exposure to the antibiotic results in a progressive decline of tissue resistance and selectivity.

The observation of no effect of amphotericin B on shunt P_{Cl}/P_{Na} disagrees with the result of Rose and Nahrwold (1976) who found an increase of P_{Cl}, as computed from the serosa-to-mucosa Cl flux and from transepithelial dilution potentials. Cremaschi *et al.* (1977), however, did not find changes of P_{Cl} over a 30-min period after exposure to amphotericin B, by essentially the same techniques, in the same tissue (rabbit gallbladder). Both groups used the same concentration of the antibiotic and the time at which the measurements were made were similar. The reason for this disagreement is unclear.

In summary, these experiments confirm and extend the recent results and conclusions of Cremaschi *et al.* (1977). Amphotericin B exerts a main effect at the luminal membrane of the gallbladder epithelial cells, reducing its K selectivity and equivalent emf. This action results in cell depolarization and transepithelial hyperpolarization. The observed changes in cell membrane resistances contribute to the increase of V_{ms}. At a period at which the effects of the antibiotic on potentials have reached a peak, paracellular P_{Cl}/P_{Na} is unchanged, but P_K/P_{Na} increases. The approach employed in this work does not allow one to rule out a role of a rheogenic basolateral Na pump. However, it is not necessary to invoke this mechanism in order to explain the action of amphotericin B.

I wish to thank M.P. Blaustein, P.J. De Weer, and A.L. Finn for their comments on a preliminary version of this manuscript. I am grateful for the technical assistance of T. Grady, and the help of S. McConnell in the preparation of the figures, and of J. Jones in

typing the manuscript. This work was supported by Grant No. AM-19580 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

Note added in proof: Rose and Nahrwold (*J. Membrane Biol.* **37**:277, 1977) have recently shown that amphotericin B increases mucosal cell membrane Na permeability (measured by unidirectional tracer influx) in rabbit and guinea pig gallbladder.

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