J. Membrane Biol. 34, 55–71 (1977)
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# Does Amphotericin B Unmask an Electrogenic $Na^+$ Pump in Rabbit Gallbladder? Shift of Gallbladders with Negative to Gallbladders with Positive Transepithelial p.d.'s

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Received 28 July 1976; revised 1 November 1976

*Summary.* When amphotericin B is added to the medium bathing the luminal side of a rabbit gallbladder preparation, a serosa positive transmural p.d. (+2 to +8 mV) arises in a few minutes.

Some authors have suggested [16] that the antibiotic would reduce tight-junction selectivity and the negative p.d. due to the backdiffusion of $Na^+$ salts from the lateral spaces: then the opposite positive p.d., created by a hypothetical electrogenic $Na^+$ pump, would be revealed. Against such an explanation, the experiments reported here show that, in parallel with the transepithelial p.d. changes, after the antibiotic addition, the luminal membrane potential is largely depolarized and the ratio between the mucosal and serosal cell resistance decreases. Moreover, the dependence on $K^+$ of the luminal membrane potential is strongly reduced. Ten minutes after the antibiotic addition, modifications of cell water, of cell ion concentrations and contents and of net water transport begin to be observed. Conversely, during the first 10-min period of treatment, no alteration in tight-junction selectivity is detectable by imposing dilution potentials across the tissue; by tracer technique a significant decrease in tight-junction selectivity is observed only 30 min after treatment.

Choline substitution for $Na^+$ completely abolishes amphotericin B effects, whereas $Cl^-$ replacement by $SO_4^{2-}$ does not affect the polyene action. As a conclusion, the primary action of the antibiotic consists of an increase of $Na^+$ conductance at the luminal cell barrier. Only a small fraction of the actual emf variation is measured across the whole epithelium because of the shunt in tight junctions.

Rabbit gallbladder develops only a slightly negative (serosa negative) transmural potential when bathed on both sides by Krebs-Henseleit solution [5, 9, 10]. This is in contrast with the majority of the absorbing epithelia which exhibit a positive transmural p.d. of some mV [2, 13, 17, 19]. Some years ago we found that in rabbit gallbladder a positive

transmural potential of some mV arises when the polyene antibiotic amphotericin B, which enhances permeability in several tissues, is added on the mucosal side [7]. This potential difference seemed to be due to an increase in $Na^+$ conductance at the mucosal barrier of the epithelial cells [5]. However, recently it has been reasonably suggested that amphotericin B would cause a decrease in tight-junction selectivity; in such a way the potential difference due to the backdiffusion of $Na^+$ salts from the lateral spaces to the lumen would be reduced and a hypothetical opposite p.d. due to an electrogenic $Na^+$ pump unmasked [16].

In this case amphotericin B could be an important tool to shed some light on the mechanism of $Na^+$ pumping in gallbladder. For this reason we have studied the p.d. induced by amphotericin B analyzing tight-junction selectivity and the intracellular electric potential. After these investigations we are able to confirm our previous results, i.e. amphotericin B increases $Na^+$ conductance through the luminal membrane of the epithelial cells and largely depolarizes the mucosal membrane potential. However, only a fraction of this potential change is detected across the whole epithelium owing to the large shunting of tight junctions. No direct effect of the antibiotic on tight-junction selectivity is revealed and the transepithelial p.d. dependent upon amphotericin B does not appear to be related to an electrogenic pump. Conversely, this p.d. compared to the change in luminal membrane potential can be useful to determine the conductance of the shunt pathway.

## Materials and Methods

### *Gallbladder Preparation*

Rabbits were killed by a blow on the head. Gallbladders were excised and washed free from bile with Krebs-Henseleit solution (sol. 1 of Table 1).

### *Determination of Net Water Transport*

The organ was cannulated as a noneverted preparation, filled with Krebs-Henseleit solution, bathed with the same solution on the seosal side and so incubated for half on hour. After incubation it was emptied, washed three times and filled with the test solution (control: Krebs-Henseleit solution) or Krebs-Henseleit solution with amphotericin B (40 μg/ml) added. Net water transport was measured gravimetrically after 5, 10, 30 or 60 min. The antibiotic dose used is twice as large as that necessary to achieve the maximal effect [7].

Table 1. Composition of the experimental salines (mM)

| Solution | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | Choline | TRIS | $HCO_3^-$ | $Cl^-$ | $SO_4^{2-}$ | $H_2PO_4^-$ | Mannitol |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bicarbonate-Krebs-Henseleit solution (sol. 1) | 142.9 | 5.9 | 2.5 | 1.2 | – | – | 24.9 | 127.7 | 1.2 | 1.2 | – |
| $Na^+$-free solution (sol. 2) | – | 5.9 | 2.5 | 1.2 | 142.9 | – | 24.9 | 127.7 | 1.2 | 1.2 | – |
| $Cl^-$ and $HCO_3^-$-free solution (sol. 3) | 135.8 | 5.9 | 2.5 | 1.2 | – | 7.1 | – | – | 77 | 1.2 | 75.8 |
| $HCO_3^-$-free solution (sol. 4) | 135.8 | 5.9 | 2.5 | 1.2 | – | 7.1 | – | 151.2 | 1.2 | 1.2 | – |
| Low NaCl solution (sol. 5) | 17.8 | 5.9 | 2.5 | 1.2 | – | 7.1 | – | 33.2 | 1.2 | 1.2 | 236.0 |

### *Determination of Cell Water and Ions*

Gallbladders opened as a flat sheet were incubated for 30 min in sol. 1 to which $^3$H-sucrose (1 μCi/ml) had been added. Then amphotericin B (40 μg/ml) was added to the medium and the tissue was incubated under such conditions for 5, 10, 30 or 60 min. In the controls the antibiotic was not added. At the end of the experiment gallbladders were blotted three times on Whatman No. 1 filter paper. Mucosa was scraped off at 0 °C and immediately weighed. Cells were broken up by osmotic shock by adding 2 ml of bidistilled water; the suspension was then frozen at −30 °C, thawed and boiled for 15 min. The supernatant was removed and the sediment dried overnight. The dry mucosa weight so obtained was subtracted from the wet mucosa weight in order to calculate mucosa total water. Duplicate samples of the supernatant and samples from the initial and final incubation medium were analyzed for radioactivity by a liquid scintillation spectrometer (TriCarb, Packard Instr. Co., Model 3315). Samples assayed for $Na^+$, $K^+$ and $Cl^-$ were deproteinized in advance with 1:1 (vol/vol) 0.6 M perchloric acid; $Na^+$ and $K^+$ were assayed by a flame spectrophotometer (Beckman DU 2); $Cl^-$ was titrated using a modified diphenilcarbazone method [8]. For further details about extracellular space determination with $^3$H-sucrose and about the calculations of cell ion concentrations *see also* references [3, 4, and 8].

### *Determination of Transepithelial $P_{Cl}$ and $P_{Na}$*

The tissue, opened as a flat sheet, was held between two lucite chambers and bathed with 8 ml of Krebs-Henseleit solution. $^{36}Cl^-$ or $^{22}Na^+$ were added to the medium on the serosal side. Transepithelial p.d. was continuously monitored. After a 2 h equilibration the mucosal medium was renewed every 30 min for six experimental periods. During the last three periods amphotericin B was added on the luminal side (40 μg/ml). Radioactivity was determined as described above; $P_{Cl}$ and $P_{Na}$ were calculated from the measured serosa to mucosa fluxes using the Nernst-Planck equation.

### *Simultaneous Determination of the Luminal Extracellular Space by Sucrose and Sulfate*

The tissue (sac preparation) was preincubated for 30 min in Krebs-Henseleit solution; it was then incubated for 60 min in sulfate solution (sol. 3 of Table 1). In the last 30 min of this second period $^3$H-sucrose (1 μCi/ml) and $^{35}SO_4^{2-}$ (0.6 μCi/ml) were added to the mucosal medium. In some experiments amphotericin B (40 μg/ml) was also present in this medium. At the end of the experiment the sac was cut open, the mucosa scraped off and processed as described above, in order to determine the luminal extracellular space as a percentage of the mucosa total water.

### *Electrical Measurements*

Gallbladders, opened lengthwise, were horizontally held between two lucite chambers, with the epithelium upwards. Serosal and mucosal chambers were respectively filled with 4 and 2 ml of saline; both solutions were continuously renewed. Luminal fluid could be rapidly changed, even when the microelectrode was held in the cell.

The schema of the electrical circuit is reported in ref. [12]. Briefly, it was possible to measure: *i*) the transepithelial p.d. ($V_{ms}$) by two calomel electrodes connected to the mucosal and serosal fluids by agar −3M KCL bridges; *ii*) the mucosal and the serosal membrane potentials ($V_m$, $V_s$) between the microelectrode in the cell and one of the two reference electrodes; *iii*) the mucosal/serosal resistance ratio ($R_m/R_s$) from $V_m$ and $V_s$ deflections when current pulses (130 μA/cm$^2$, 1 sec.) were passed through the tissue; *iv*) the tissue and epithelium resistance ($R_t$, $R_{ep}$) from the $V_{ms}$ deflection for the passage of the same current pulses. A correction to estimate the actual potential drop across membranes during pulses ($\Delta V_m$, $\Delta V_s$, $\Delta V_{ms}$) was obtained by measuring the p.d. between mucosal bridge and microelectrode, just before impalement or after penetrating the serosal membrane (that was acknowledged by the return of the recorded potential to the baseline). The correction to estimate the actual potential drop across the total tissue was obtained by measuring the p.d. between the two bridges without the tissue being present. For further details *see* ref. [12].

### *Dilution Potential Measurements*

In order to measure dilution potentials across the epithelium, the tissue was vertically held between two lucite chambers. A simplified saline without bicarbonate, buffered with Tris (pH 7.4) was used. This solution was on the serosal or on the mucosal side: on the opposite side either the same solution or a saline with 118 mM NaCl isosmotically replaced with mannitol (sol. 5 of Table 1) was present. Both media were continuously oxygenated (100% $O_2$) and vogorously stirred by magnetic stirrers. Basal transepithelial p.d. was measured in advance; then, a dilution potential serosa to mucosa or vice-versa was created and, when it was steady, amphotericin B (40 μg/ml) was added to the luminal medium. The new constant value of dilution p.d. achieved was measured; then both solutions were restored with sol. 4 on both sides (the polyene was always present on the mucosal side). Under these conditions the transepithelial p.d. evoked by amphotericin B was determined. Since only comparisons of dilution potentials before and after amphotericin B addition were considered, no correction was applied for the possible slight junction potentials between agar-KCl bridges and test solutions.

## Results

### *Transepithelial p.d., Net Water Transport, Intracellular Potential, Water and Ions*

When the tissue is bathed with Krebs-Henseleit solution on both sides, membrane potentials across the mucosal ($V_m$) and serosal ($V_s$) barrier of the cell are nearly equal ($\sim -59$ mV), so that a transepithelial potential difference of only $-0.44$ mV (serosa negative) is measured (Table 2a). This is in complete agreement with data previously reported [12].

If we now add amphotericin B to the mucosal medium (40 μg/ml), in a few minutes a transepithelial p.d. equal to $+3.2$ mV arises, as previously seen [5, 7]. In parallel, a large decrease in luminal membrane

Table 2. Electrical parameters under control conditions and under amphotericin B treatment in various solutions

| Solution | | $V_{ms}$ (mV) | $V_m$ (mV) | $R_m/R_s$ | $R_{mK}/R_s$ | $\Delta V_{mK}$ (mV) | $\Delta E_{mK}$ (mV) |
|---|---|---|---|---|---|---|---|
| (a) Bicarbonate-Krebs-Henseleit solution | Control: | $-0.44$ | $-58.6$ | 0.98 | 1.03 | 15.8 | 32.4 |
| | | $\pm 0.33$ | $\pm 0.4$ | $\pm 0.06$ | $\pm 0.13$ | $\pm 0.8$ | $\pm 1.9$ |
| | | (5) | (214–6) | (6–2) | (11–4) | (19–5) | (19–5) |
| | Amphotericin B | $-3.20$ | $-32.4$ | 0.50 | 0.51 | 10.9 | 15.3 |
| | | $\pm 0.70$ | $\pm 2.0$ | $\pm 0.07$ | $\pm 0.16$ | $\pm 1.1$ | $\pm 1.4$ |
| | | (4) | (40–4) | (11–1) | (8–4) | (8–4) | (8–4) |
| (b) $Cl^-$-free solution | Control: | $-1.10$ | $-58.7$ | 0.84 | 0.99 | 18.0 | 36.2 |
| | | $\pm 0.70$ | $\pm 0.9$ | $\pm 0.15$ | $\pm 0.10$ | $\pm 0.9$ | $\pm 2.9$ |
| | | (3) | (58–3) | (5–2) | (7–3) | (10–3) | (10–3) |
| | Amphotericin B | $+3.00$ | $-36.8$ | — | 0.55 | 11.2 | 17.9 |
| | | $\pm 0.10$ | $\pm 1.3$ | | $\pm 0.10$ | $\pm 1.2$ | $\pm 2.9$ |
| | | (3) | (21–3) | | (7–3) | (9–3) | (9–3) |
| (c) $Na^+$-free solution | Control: | $-1.00$ | $-58.1$ | 0.78 | 1.00 | 16.6 | 33.2 |
| | | $\pm 0.20$ | $\pm 0.8$ | $\pm 0.07$ | $\pm 0.25$ | $\pm 0.7$ | $\pm 1.3$ |
| | | (5) | (120–7) | (6–2) | (10–2) | (20–4) | (26–5) |
| | Amphotericin B | $-1.40$ | $-56.4$ | — | 0.76 | 19.5 | 34.0 |
| | | $\pm 0.50$ | $\pm 0.9$ | | $\pm 0.15$ | $\pm 1.2$ | $\pm 2.2$ |
| | | (4) | (44–4) | | (5–1) | (12–4) | (12–4) |

The antibiotic (40 μg/ml) was added to the luminal medium. $V_{ms}$ transmural p.d.; $V_m$=mucosal membrane p.d.; $R_m/R_s$=mucosal/serosal resistance ratio; $R_{mK}/R_s$=mucosal/serosal resistance ratio when $[K]_m^+$ is raised to 24.7 mM; $\Delta V_{mK}$=mucosal membrane p.d. change when $[K]_m^+$ is raised to 24.7 mM; $\Delta E_{mK}$=mucosal membrane emf change when $[K]_m^+$ is raised to 24.7 mM. All of the values are reported as mean ± SE (the number of impalements and of gallbladders are reported in parentheses).

Table 3. Cell water and net water transport versus time under control conditions (*C*) and under amphotericin B treatment (*E*) in bicarbonate Krebs-Henseleit solution

| Time (min) | Cell water/dmw[a] | | Net water transport (μl $cm^{-2}$ $h^{-1}$) | |
|---|---|---|---|---|
| | *C* | *E* | *C* | *E* |
| 0 | 8.7 ± 1.3 (4) | – | – | – |
| 5 | – | 8.4 ± 0.6 (9) | – | – |
| 10 | – | 8.3 ± 0.8 (6) | – | 85.4 ± 19.9 (6) |
| 30 | 7.4 ± 0.4 (15) | 10.5 ± 0.7 (10) | 86.9 ± 8.9 (16) | 41.5 ± 5.9 (6) |
| 60 | 7.7 ± 0.7 (11) | 10.6 ± 0.9 (13) | 88.6 ± 6.7 (14) | 30.6 ± 6.7 (9) |

The antibiotic (40 μg/ml) was added to the luminal medium (time 0) after a 30-min preincubation. Cell water is expressed as mg of water/mg of dry mucosa weight. Values are reported as mean ± SE with the number of experiments in parentheses.
[a] dry mucosa weight.

potential is recorded (from −58.6 to −32.4 mV; *see* Table 2a). A similar change in serosal membrane potential must occur, since transepithelial p.d. is only +3.2 mV.

These measurements were carried out within 10 min of the addition of the antibiotic; in this time cell water and net water transport do not appear significantly modified (Table 3), so that neither cell swelling nor changes in intercellular channel width are reasonably supposed to occur. In accordance with the absence of a cell swelling also $Na^+$, $K^+$ and $Cl^-$ contents and concentrations remain constant during this period (Fig. 1*a* and *b*). Subsequently an increase in cell $Na^+$ content and concentration and a decrease in $K^+$ concentration without any change in its content are observed. In parallel, cell water increases following $Na^+$ entry and this explains $K^+$ dilution. Cell $Cl^-$ concentration is not significantly modified by the cell swelling as the $Cl^-$ content increases (Table 3; Fig. 1*a* and *b*).

### *Transmural $P_{Na}$ and $P_{Cl}$*

$Na^+$ and $Cl^-$ permeabilities, measured by serosa to mucosa fluxes, every 30 min, are reported in Fig. 2. No significant increase is detected for both ion permeabilities in the first 30-min period after treatment, whereas, subsequently, both become larger, $P_{Cl}$ relatively much larger than $P_{Na}$.

### *Plasma Membrane Electrical Resistances*

If potential drops on luminal and serosal barriers ($\Delta V_m$, $\Delta V_s$), caused by a current pulse passed through the tissue, are measured and the mucosal/serosal resistance ratio ($R_m/R_s$) is calculated, a value nearly equal to 1 is obtained (*see* Table 2a); this value is equal to that previously reported [12].

When amphotericin B is added to the luminal medium (40 µg/ml) the ratio rapidly drops from 0.98 to 0.50. This drop could not be explained by an increase of the basolateral resistance due to a closure of the lateral spaces, as no change in lateral space width would occur, at least in the first 10-min period after the antibiotic addition, as above pointed out. So a decrease of the luminal resistance is likely to take place. Further support of this explanation is given by the fact that, in the first 10-min period, with equal pulses, $\Delta V_m$ is smaller and $\Delta V_s$ is larger than under control conditions (as can be predicted on the basis of a decrease in $R_m$ and of the consequent increase in the electrical current through the cell).

### *$V_m$ Dependence on $K^+$*

Under control conditions the mucosal membrane potential ($V_m$) is largely dependent on $K^+$ and, to a lesser extent, quantitatively variable with species, to $Na^+$ concentration [12, 15, 20]. Thus, if luminal $K^+$ concentration is increased from the physiological value (5.9 mM) to 24.7 mM (by substituting $K^+$ for $Na^+$ and by maintaining constant the anion concentration) a large and immediate depolarization of the luminal membrane is obtained ($\Delta V_m = 15.8$ mV; *see* ref. [12] and Table 2a). The measurement is carried out by maintaining the microelectrode in the cell during the solution replacement[1]. On the basis of this value and

1 A $V_m$ dependence on luminal $K^+$ concentration has been observed also in *Necturus* gallbladder, although to a lesser extent than for rabbit [12, 15, 20]. It is very unlikely that the $V_m$ change taken into account is due to $K^+$ which, by diffusing through the tight junctions, would alter the intercellular channel $K^+$ concentration and the serosal emf. In fact, the $V_m$ change upon $[K^+]_m$ variations is immediate and achieves a maximum in a few (5–15) seconds [12]. A change in $K^+$ concentration around the basolateral membrane as a consequence of a $[K^+]_m$ variation should cause a slow and continuous $V_m$ depolarization until a concentration profile in steady state is obtained in the tissue (and this requires a time of many minutes, *see* e.g. ref. [9]. As a matter of fact sometimes a slow drift of the maximum was observed, but it can be due to many causes and not only to that reported above; anyway in this case the impalement was discarded.

Moreover, since tight-junctions are the site of maximal resistance in the paracellular pathway of the tissue, when lateral spaces are open [18], the maximal concentration drop upon the $[K^+]_m$ change must occur across them.

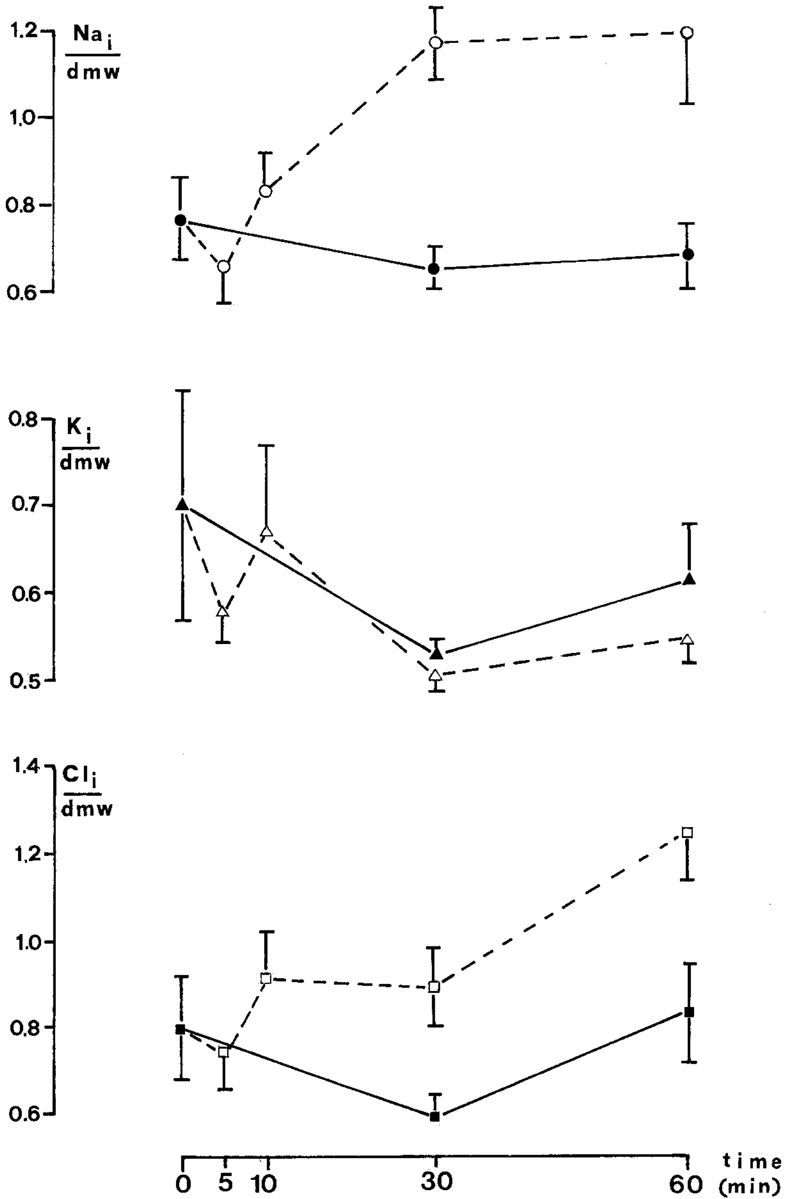


Fig. 1a

Fig. 1. $Na^+$, $K^+$ and $Cl^-$ intracellular contents (*a*) and concentrations (*b*) under control conditions (solid symbols) or under amphotericin B treatment (open symbols) versus time. The incubation was carried out in bicarbonate-Krebs-Henseleit solution; the antibiotic (40 μg/ml) was added to the luminal medium (time 0) after a 30-min preincubation. Ion contents are expressed as μmoles/mg of dry mucosa weight. Each value is mean± SE of 10 experiments (only the value reported for control at 30 min is the mean of 17 experiments)

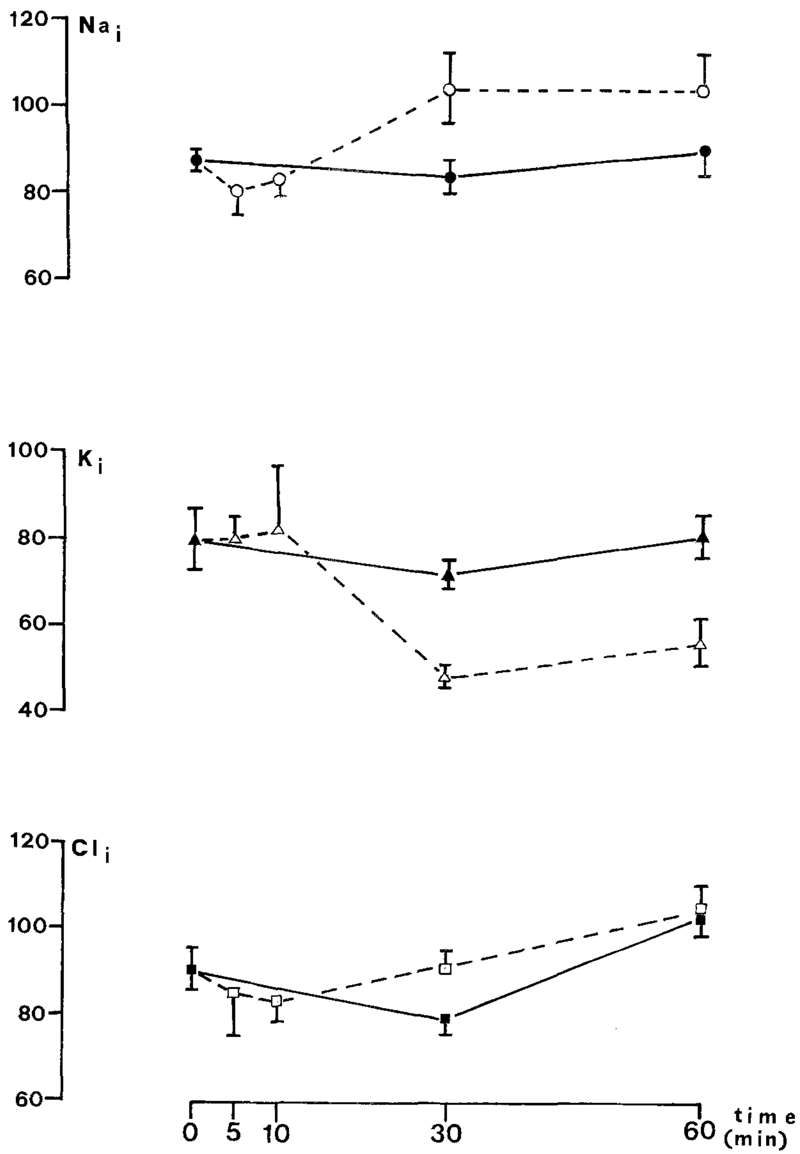


Fig. 1b

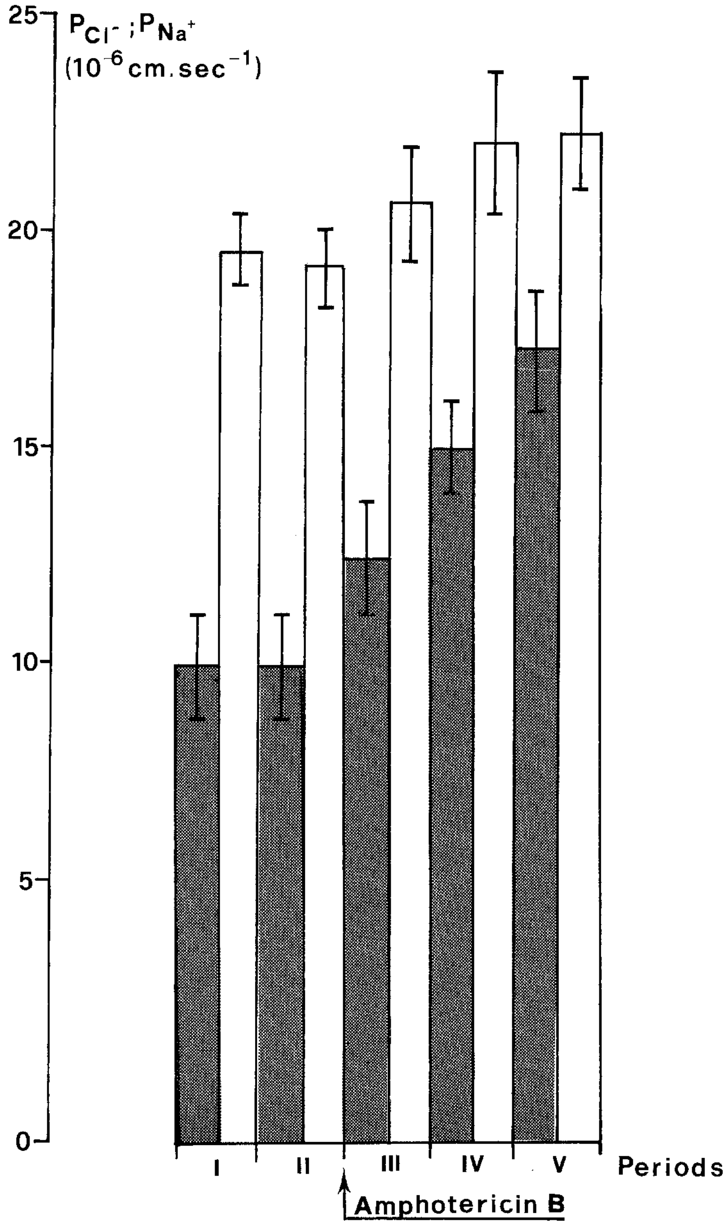


Fig. 2. $Cl^-$ and $Na^+$ transepithelial permeabilities measured in bicarbonate-Krebs-Henseleit solution under control conditions and under amphotericin B treatment. The antibiotic (40 µg/ml) was added after two experimental periods (arrow) to the luminal medium. Each experimental period was 30 min. $P_{Cl}$ (eight experiments) and $P_{Na}$ (22 experiments) are represented by grey and white columns, respectively. Histograms are means ± SE

of the equation reported in ref. [12], which corrects for the shunting effect of tight-junctions[2], one can calculate 32.4 mV of actual change in luminal emf ($\Delta E_{mK}$) (Table 2a). The resistance ratio applied for this correction is that measured when 24.7 mM $K^+$ is in the luminal medium ($R_{mK}/R_s$). Both $R_{mK}/R_s$ and $\Delta V_{mK}$, and as a consequence $\Delta E_{mK}$, are not significantly different from those previously reported [12].

If these measurements are repeated in the first 10-min period after treatment, a significant decrease of the three parameters is observed (Table 2a). The luminal membrane potential seems now less dependent on $K^+$ concentration.

### *$Cl^-$ $HCO_3^-$ Replacement by $SO_4^{2-}$ in the Incubation Fluids*

When the latter experiment is repeated by using mucosal and serosal incubating fluids in which $SO_4^{2-}$ is substituted for $Cl^-$ and $HCO_3^-$ (sol. 3 of Table 1) the obtained data (Table 2b) are not significantly different with respect to those obtained in Krebs-Hanseleit solution. Under control conditions this result was well known: on this basis and on other evidence [12] the luminal cell membrane was considered to exhibit no $Cl^-$ conductance. It is to emphasize that under control conditions as well as under amphotericin B treatment $SO_4^{2-}$ does not enter the cell[3]. Now, if we compare Table 2a with Table 2b, we can observe that also under antibiotic treatment, both by incubating the tissue in $SO_4^{2-}$ and $Cl^-$-$HCO_3^-$ solutions, the same values of all of the reported parameters ($V_{ms}$, $V_m$, $R_{mK}/R_s$, $\Delta V_{mK}$, $\Delta E_{mK}$) are obtained. Thus $Cl^-$ and $HCO_3^-$ do not seem to be responsible for the large change in conductance of the luminal membrane induced by amphotericin B.

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2 The equation used is:

$$\Delta E_{mK} = \Delta V_{mK} \left(1 + \frac{R_{mK}}{R_s}\right).$$

For derivation *see* ref. [12]. It does not take into account an emf in the tight junction, since in our type of experiments this emf is negligibly activated, as can be observed in ref. [12]. In fact, $[K^+]_m$ changes are carried out maintaining a large $Na^+$ concentration in both mucosal and serosal media. This shortcircuits $K^+$ dilution potentials at the tight junctions, owing to the internal shunting effect of $Na^+$ in that pathway. Significant biionic potentials are observed in gallbladder only when $K^+$ is largely substituted for $Na^+$ in one of the two media [*see* e.g. J.H. Moreno and J.M. Diamond. *J. Membrane Biol.* (1974) **15**:277].

3 The values of the luminal extracellular space obtained with $^{35}SO_4^{2-}$ or $^3H$-sucrose appear to be equal. Under control conditions they are $8.9 \pm 1.6\%$ (8 exp.) and $6.2 \pm 1.3\%$ (8 exp.), respectively. Under amphotericin B treatment (40 µg/ml added to the luminal medium) they are $7.7 \pm 1.1\%$ (4 exp.) and $9.5 \pm 2.4\%$ (4 exp.). These values are reported as percentage of the mucosa total water (*see also* ref. [3, 4]).

### *$Na^+$ Replacement by Choline in the Incubating Fluids*

When in the mucosal and serosal incubating fluids $Na^+$ is replaced by choline (sol. 2 of Table 1) no change in $V_{ms}$, $V_m$, $R_m/R_s$, $R_{mK}/R_s$, $\Delta V_{mK}$, $\Delta E_{mK}$ is observed (Table 2c) with respect to the values reported in Table 2a. Such a result completely confirms previous data [12].

If amphotericin B is added in the lumen to choline solution not one of the antibiotic effects is now exhibited (Table 2c): $V_{ms}$ does not arise, $V_m$ and $R_m/R_s$ do not decrease and the dependence on $K^+$ of the luminal membrane potential remains normal.

As a conclusion, the antibiotic effect on the luminal membrane appears essentially related to an increase of $Na^+$ conductance.

### *Transepithelial Dilution Potentials*

When luminal NaCl concentration is decreased by replacing 118 mM NaCl with mannitol, a transepithelial p.d. (serosa negative) is elicited (Table 4: exp. no. 1, 2, 3, 4). If amphotericin B is added to the luminal medium, this p.d. is reduced. The decrease is not larger than the transepi-

Table 4. Spontaneous transepithelial p.d.'s and dilution potentials under control conditions and amphotericin B treatment

| Solution no. | | Transepithelial p.d. (mV) | | | |
|---|---|---|---|---|---|
| Lumen | Blood side | Exp. no. 1 | Exp. no. 2 | Exp. no. 3 | Exp. no. 4 |
| 4 | 4 | −0.10 | −0.01 | +0.60 | −0.55 |
| 5 | 4 | −11.50 | −18.00 | −22.00 | −24.00 |
| 5 +Amphot. | 4 | −8.50 | −16.50 | −19.00 | −20.00 |
| 4 +Amphot. | 4 | +2.90 | +4.00 | +4.50 | +5.00 |
| | | Exp. no. 5 | Exp. no. 6 | | |
| 4 | 4 | −0.01 | −0.20 | | |
| 4 | 5 | +17.00 | +16.00 | | |
| 4 +Amphot. | 5 | +20.00 | +17.00 | | |
| 4 +Amphot. | 4 | +4.00 | +5.00 | | |

The antibiotic (40 µg/ml) was added to the luminal medium. Dilution potentials were evoked by bathing the tissue on one side with a low $Na^+$ solution (sol. 5) and on the opposite side with sol. 4.

thelial p.d. evoked by the antibiotic when the tissue is bathed on both sides by identical Krebs-Hanseleit solutions.

If the experiment is repeated by reducing NaCl concentration (sol. 5) on the serosal side and by using sol. 4 as mucosal medium, a serose positive transepithelial p.d. is elicited. When amphotericin B is added to the lumen, this transepithelial p.d. increases a few mV (Table 4: exp. no. 5, 6).

Thus, the antibiotic does not reverse or univocally change transepithelial dilution potentials.

## Discussion

### *Does the Antibiotic Act on Tight-Junctions or on the Luminal Cell Membrane?*

The transepithelial p.d. created by amphotericin B added to the luminal medium ($V_{msA}$), when both sides of gallbladder are bathed by the same solution, is about 3 mV (the antibiotic added to the serosal medium apparently does not modify the transepithelial p.d.; *see* ref. [7]). If $V_{msA}$ is caused by a change in selectivity of tight-junctions, it should be found distributed on the luminal and basolateral resistances of the cell. Thus, across each barrier, after the antibiotic treatment, a change in membrane potential should arise less than the transepithelial change. Since $V_{msA}-V_{ms}=+3.2-(-0.44)=+3.6$ mV and $R_m/R_s$ is 0.50 we should measure a 1.2-mV change across the luminal barrier and a 2.4-mV change across the serosal barrier. On the contrary we measure 26.2 and 29.4-mV changes, respectively; i.e. changes 10 times larger than the predicted ones. This is consistent only with the hypothesis that amphotericin B directly acts on the luminal membrane, modifies its conductance and causes a large depolarization (some 10 mV) of the luminal emf ($\Delta E_{mA}$). In this case such a potential change creates an electrical current through cell resistances and the shunt pathway, so that: *i*) the serosal membrane potential also changes ($\Delta V_{sA}=i \cdot R_s$), *ii*) the measured change in the mucosal membrane potential ($\Delta V_{mA}$) is less than the mucosal emf ($\Delta E_{mA}$), ($\Delta V_{mA}=\Delta E_{mA}-i.R_m$), *iii*) the measured change in the transepithelial p.d. ($\Delta V_{msA}$) is only a small fraction of $\Delta E_{mA}$ ($\Delta V_{msA}=\Delta V_{mA}-\Delta V_{sA}=\Delta E_{mA}-i \cdot R_m-i \cdot R_s$).

The other data reported here are consistent with such an explanation. At least 10 min after the treatment the potential changes are followed by changes in cell water and ions: the cell swells, cell $Na^+$ increases and cell $K^+$ decreases; $Na^+$ and $Cl^-$ contents increase. The unbalance

of the ion cell concentrations can cause the inhibition of the isotonic net water transport (*see also* ref. [6]). Furthermore, immediately after the antibiotic treatment, the ratio between the mucosal and the serosal cell resistances decreases, in parallel with the change in $V_{ms}$ and in $V_m$. Since initially no closure of the lateral spaces is observed (*see* Results), the ratio decrease should be ascribed to an $R_m$ decrease rather than to an $R_s$ increase. Moreover, the dependence on $K^+$ of the luminal potential ($V_m$) and emf ($E_m$) is largely reduced by amphotericin B; this is further evidence that the antibiotic causes a change in conductance of the luminal membrane.

At last, an immediate direct action of amphotericin B on tight junctions is also excluded by the analysis of the transepithelial dilution potentials created by lowering NaCl on one side of the tissue (it is well known that they arise in tight junctions; *see* e.g. ref. [1]). Let us suppose that the serosa positive $\Delta V_{msA}$ is due to a change induced by the antibiotic in the fixed charges at the junctions (from neutral/negative to positive fixed charges); the small negative transepithelial p.d. measured under control conditions and due to a backdiffusion of $Na^+$ salts from the lateral spaces, then reverses. However, in this case, also the dilution potentials, artificially imposed across the epithelium, would reverse their sign. Conversely, after the antibiotic addition, by lowering [NaCl] in the lumen we observe a negative p.d. and by lowering [NaCl] on the serosal side we measure a positive p.d. as well as before amphotericin B treatment.

If the antibiotic would merely reduce tight-junction selectivity, both the mucosa to serosa and the serosa to mucosa dilution potentials should be reduced. On the contrary the latter only is decreased by the treatment, whereas the former is increased. Furthermore, in order to account for the 3–5.5 mV of $\Delta V_{msA}$ the reduction of the serosa to mucosa dilution potentials, experimentally induced, should be quite larger than the measured 1.5–4 mV. In fact, the applied concentration difference ($\Delta C_{NaCl}$ = 118 mM) is quite larger than that present across tight junctions under apparently isosmotic conditions (~40 mM NaCl as a maximal value, ref. [14]).

Also measurements of $P_{Cl}$ and $P_{Na}$ by serosa to mucosa fluxes, which, as well as transepithelial electrical resistance and dilution potentials, are mainly related to the shunt pathway [18], confirm that in the first 30-min period upon treatment no significant change in tight-junction selectivity is detectable. In contrast, it is evident that after this period, both $P_{Cl}$ and $P_{Na}$ increase, the former more than the latter, so that

the transmural ion selectivity decreases. Probably the cell swelling damages in some way the tissue: as a matter of fact $P_{Cl}/P_{Na}$ ratio increases towards the value reported for free diffusion.

### *Action of Amphotericin B on the Luminal Cell Membrane*

$Cl^-$ replacement by $SO_4^{2-}$ does not seem to affect the amphotericin B action on any parameter. Thus, the positive $\Delta V_{msA}$ normally arises; $V_m$, $E_m$, $R_m/R_s$ are reduced to the usual extent; $E_m$ dependence on $K^+$ is also normally decreased. Since $Cl^-$, with or without amphotericin B affecting the epithelium, is never distributed between the incubation medium and the cell according to a membrane electrochemical equilibrium (*see* Table 2 and Fig. 2), we must conclude, in order to explain the effects of its replacement by $SO_4^{2-}$, that the cell does not exhibit any $Cl^-$ conductance after treatment with the antibiotic as well as before. On the contrary, $Na^+$ replacement with choline completely abolishes any amphotericin B effect on the electrical parameters. So, the polyene seems to modify the luminal membrane by largely increasing $Na^+$ conductance: for this reason $R_m$, $E_m$ and $E_m$ dependence on $K^+$ decrease.

This conclusion is not inconsistent with that previously reported, deduced by the mere analysis of $\Delta V_{msA}$ [5]. In that case, we suggested an increase in conductance also for $Cl^-$, although to a lesser extent than for $Na^+$. The experiment was performed by evoking $\Delta V_{msA}$ and then substituting $SO_4^{2-}$ for $Cl^-$. With such a protocol $\Delta V_{msA}$ appeared abolished upon substitution. This abolition can now be explained by a masking effect on the positive $\Delta V_{msA}$ of the transient negative transepithelial p.d. due to NaCl trapped in the large serosal unstirred layers, when serosal and mucosal media are already completely changed. As a matter of fact, in the present work the experiment was entirely carried out in $SO_4^{2-}$ solution and the antibiotic addition, in spite of the absence of $Cl^-$, evoked a typical $\Delta V_{msA}$.

### *Transepithelial Electrical p.d. and Ion Selectivity of the Luminal Membrane*

The reported data show that an increase in $Na^+$ conductance of the luminal membrane causes a positive transepithelial p.d. Recently, it has been reported that, in some animal species, gallbladders exhibit positive transmural p.d.'s [11]. It seems likely that in different gallbladders

different extents of luminal $Na^+$ conductance are present, so that $E_m$ and $E_s$ can be nearly equal or very different and $V_{ms}$ can be nearly zero or largely positive (e.g. in man). As a matter of fact, in rabbit gallbladder with $V_{ms}$ nearly zero or slightly negative $E_m$ and $E_s$ seem nearly equal [12]; in *Necturus* gallbladder, which exhibits a positive $V_{ms}$ [15, 20] $E_m$ seems smaller than $E_s$ because of a consistent $Na^+$ luminal conductance.

Then, amphotericin B, in adequate dose (for the dose-response correlation *see* ref. [7]), can shift the characteristics of a rabbitlike gallbladder towards those of a manlike gallbladder.

We are much indebted to Prof. V. Capraro for helpful discussion. This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Rome.

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