

The Water Permeability of Toad Urinary Bladder

I. Permeability of Barriers in Series with the Luminal Membrane

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ABSTRACT Antidiuretic hormone (ADH) induces a large increase in the water permeability of the luminal membrane of toad urinary bladder. Measured values of the diffusional water permeability coefficient, $P_d(w)$, are spuriously low, however, because of barriers within the tissue, in series with the luminal membrane, that impede diffusion. We have now determined the water permeability coefficient of these series barriers in fully stretched bladders and find it to be $\sim 6.3 \times 10^{-4}$ cm/s. This is equivalent to an unstirred aqueous layer of ~ 400 μm . On the other hand, the permeability coefficient of the bladder to a lipophilic molecule, hexanol, is $\sim 9.0 \times 10^{-4}$ cm/s. This is equivalent to an unstirred aqueous layer of only 100 μm . The much smaller hindrance to hexanol diffusion than to water diffusion by the series barriers implies a lipophilic component to the barriers. We suggest that membrane-enclosed organelles may be so tightly packed within the cytoplasm of granular epithelial cells that they offer a substantial impediment to diffusion of water through the cell. Alternatively, the lipophilic component of the barrier could be the plasma membranes of the basal cells, which cover most of the basement membrane and thereby may restrict water transport to the narrow spaces between basal and granular cells.

INTRODUCTION

The antidiuretic hormone (ADH)-induced increase in the water permeability of toad urinary bladder has interested numerous investigators from such diverse disciplines as biophysics, cell biology, and renal physiology. Over the years certain facts have emerged: (a) the hormone action is mediated through a second messenger, 3',5' cyclic AMP (Handler and Orloff, 1973); (b) the increased water permeability of the epithelium as a whole reflects a specific increase of water

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permeability of the mucosal (luminal) membrane of the granular epithelial cells (Di Bona et al., 1969); (c) this increased water permeability occurs through pores or channels (Finkelstein, 1976) that (d) are probably inserted into the luminal membrane by fusion with it of cytoplasmic tubular vesicles containing the channels (Muller et al., 1980; Wade, 1980); (e) these channels are permeable to water and little else—small ions and nonelectrolytes are essentially impermeant (Finkelstein and Rosenberg, 1979; Grantham and Burg, 1966; Carvounis et al., 1979).

A long-standing issue, arising well before any of the above features of ADH action were known, has been the ratio of the osmotic water permeability coefficient, P_f , to the diffusional water permeability coefficient, $P_d(w)$, in ADH-stimulated tissues. The value of $P_f/P_d(w)$ is of interest because significant inferences concerning the physical nature of the water permeation pathway can be made from it (see Levine et al., 1984, for a discussion of this point). It has been apparent for some time, however, that measured values of $P_f/P_d(w)$ in ADH-stimulated tissues greatly overestimate the true value in the luminal membrane. This is because series barriers within the tissue and unstirred aqueous layers in the experimental chambers conspire to make measured values of $P_d(w)$ spuriously low (Hays and Franki, 1970). Previous attempts to correct measured values of $P_d(w)$ for these factors have not, in our opinion, been particularly successful or convincing (e.g., Schafer and Andreoli, 1972; Hebert and Andreoli, 1980).¹ We now enter the lists and report, in this and the following paper (Levine et al., 1984), the results of experiments directed at determining the true value of $P_f/P_d(w)$ in ADH-stimulated and 8-bromo cyclic AMP-stimulated toad urinary bladders.

In this paper we characterize the series barriers to water (THO) diffusion within the toad urinary bladder. We first describe our experimental procedure for measuring P_f and $P_d(w)$ and show that unstirred aqueous layers within the well-stirred chamber offer a trivial resistance to THO diffusion. We then describe two experimental methods for determining the resistance of the series barriers within the bladder to THO diffusion, and report good agreement between the two. We also determine the resistance of these series barriers to diffusion of a lipophilic molecule, hexanol, and compare it with their resistance to THO diffusion. Our analysis of series barriers and unstirred aqueous layers provides a method for correcting measured values of $P_d(w)$ to obtain true values of $P_d(w)$ in the luminal membrane. In the following paper we apply this method to determining $P_f/P_d(w)$ for the water permeation pathway induced in the luminal membrane by ADH and 8-bromo cyclic AMP.

¹ The analysis of Hebert and Andreoli (1980) leads to the physically unlikely conclusion that the luminal membrane presents a substantial permeability barrier to butanol, a small lipophilic molecule. The use of lipophilic solutes to estimate series barrier resistances to water (Schafer and Andreoli, 1972) is criticized in the Discussion. The approach by Hays and Franki (1970) corrects for series barriers in subepithelial supporting structures and unstirred aqueous layers in their experimental chamber, but does not (as they acknowledge) correct for series barriers within the epithelial layer.

MATERIALS AND METHODS

Millipore Filter Experiments

HIGH-SPEED CHAMBER The design of this chamber (Fig. 1) was based on one used by Peterson and Gregor (1959) to reduce unstirred layers to $<10 \mu\text{m}$. The chamber consisted of two lucite compartments, each having a thin face with a 6.3-mm-diameter (31 mm^2) hole in it. When a millipore filter (Millipore Corp., Bedford, MA) was clamped between the two faces, it occluded the pathway between the two compartments, so that any transport between them occurred through the millipore membrane. Each compartment was stirred at 3,450 rpm by a 1.5-cm-diameter propeller that swept the entire membrane area with a linear velocity of 270 cm/s at a distance of 1–2 mm from the membrane surface. This degree of stirring had been shown to reduce unstirred layers to $\sim 4 \mu\text{m}$ (Peterson and Gregor, 1959), and our own experiments bore this out (see Results).

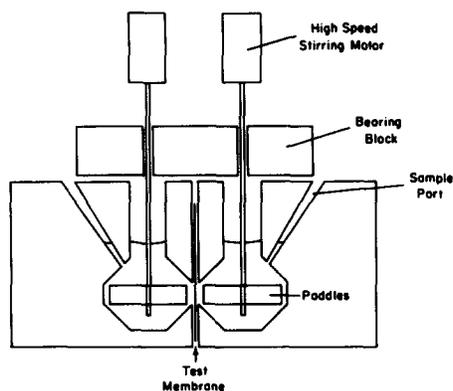


FIGURE 1. High-speed chamber designed to minimize unstirred layers. See text for details.

$P_d(w)$ and $P_d(\text{hexanol})$ were simultaneously determined in each experiment. THO and [^{14}C]hexanol (purchased from New England Nuclear Corp., Boston, MA) were added from concentrated stock solutions (1 mCi/ml and 50 $\mu\text{Ci/ml}$, respectively) to one compartment to a final concentration of ~ 1 and 0.1 $\mu\text{Ci/ml}$, respectively, and 50- μl samples were taken every 2 min from both compartments for counting in a liquid scintillation counter. P_d 's were calculated from the defining equation:

$$P_d = \frac{\Phi^*}{A \Delta c^*} \quad (1)$$

where Φ^* = flux of tracer across the membrane; A = membrane area; Δc^* = difference in concentration of tracer in the two compartments.

TRANSPORT CHAMBER $P_d(w)$ and $P_d(\text{hexanol})$ were also simultaneously measured in the chamber used for toad bladder experiments. This chamber, called the "transport chamber," is described below. The experimental procedure was essentially the same as that described above. By comparing P_d values determined for the filter in the transport chamber with those determined in the high-speed chamber, we calculated the unstirred layer thickness in the former (see Results). All experiments in both the high-speed and transport chamber were performed at room temperature.

Toad Bladder Experiments

P_d (of both H_2O and hexanol) and P_f were simultaneously determined at room temperature in the transport chamber shown in Fig. 2. Fully distended hemibladders from the toad *Bufo marinus* (obtained from National Reagents Co., Bridgeport, CT) were washed several times in amphibian phosphate Ringer's solution (110 mM Na^+ , 0.5 mM Ca^{++} , 4 mM K^+ , 106 mM Cl^- , 5 mM phosphate, pH 7.4, 220 mosmol) and then mounted fully stretched across the 2.9-cm-diameter opening of the lower compartment with their mucosal (luminal) surface down; the upper compartment with its support grid (see below) was then fastened in place above it. Parafilm gaskets were used to prevent edge damage to the tissue. The 8-ml mucosal bath was stirred at 400–500 rpm by a cylindrical magnetic stirring bar, slightly shorter than the diameter of the lower compartment, which swept within 1–2 mm of the mucosal surface. The 8-ml serosal bath was stirred at 800 rpm by a directly driven

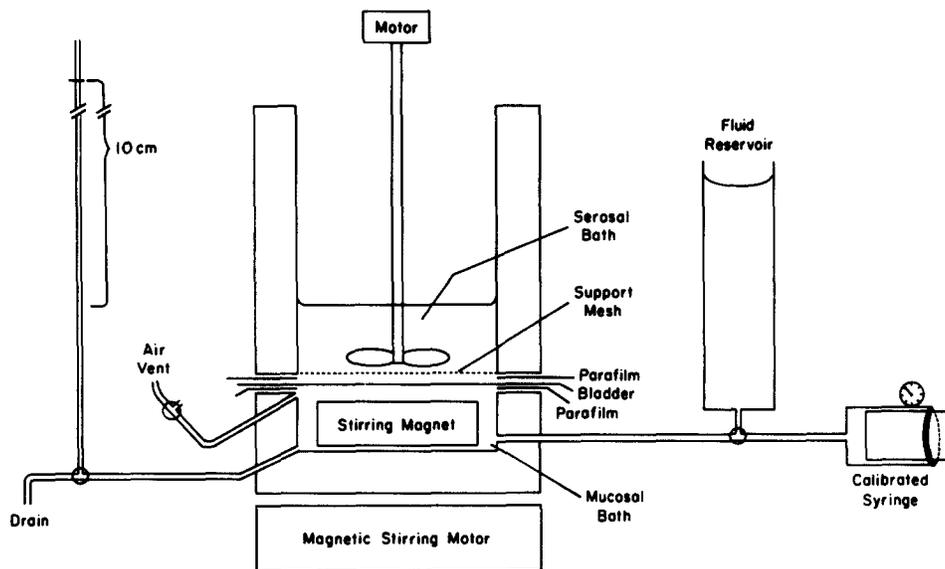


FIGURE 2. Transport chamber for simultaneous measurement of osmotic and diffusional water transport. See text for details.

plastic propeller, of similar diameter to the magnetic stirring bar, which swept within 1–2 mm of the serosal surface. Fluids of appropriate osmolality containing the desired test isotopes could be accurately added to the mucosal bath through a series of ports with a calibrated syringe readable to $0.2 \mu l$; the serosal bath was directly accessible.

The lower compartment (mucosal bath) was a closed compartment, except for a thin vertical tube (1.1 mm diameter) with a fluid level 10 cm above the open, serosal level. The tissue was prevented from bulging under this hydrostatic pressure into the serosal bath by a support grid contacting the serosal surface. The grid was of 5-0 stainless steel (diameter of 0.127 mm) woven at a 2.5-mm spacing and stabilized by a diagonal network of 7-0 nylon (diameter 0.063 mm) knotted to the steel grid at points of intersection. The grid was fastened to the opening of the upper chamber with epoxy cement. From the wire diameters and their spacing, we calculated that the area occluded by the grid was only 12% of the chamber opening. (Diffusional permeabilities measured on tissues sup-

ported by this grid were almost twice those measured on tissues supported by commercial screening [40–50% open area], fabric, or thin filter paper, no doubt because the latter group occluded a significant portion of the serosal surface.)

A typical experiment to determine series barrier permeabilities to H₂O and hexanol proceeded as follows: after the bladder was mounted in the chamber, the mucosal compartment was filled with dilute Ringer's solution (one part Ringer's solution plus four parts distilled water) containing THO and [¹⁴C]hexanol at concentrations of ~1 and 0.1 μCi/ml, respectively; the serosal compartment was filled with undiluted Ringer's solution. After 10–15 min, when the tissue had achieved a stable position on the support grid, the serosal bath was replaced with fresh Ringer's solution and stirring was begun. Water flows were measured at 4-min intervals by infusing sufficient solution into the mucosal compartment to maintain the fluid level in the thin vertical tube 10 cm above the serosal level. 50-μl samples were taken at 2-min intervals from the serosal bath for counting, and the removed volume was replaced by Ringer's solution. After 15 min of data was obtained for this basal period, vasopressin (synthetic grade V from Sigma Chemical Co., St. Louis, MO) was added to the serosal bath to a maximally stimulating concentration of 20 mU/ml, and sampling and flow measurements were continued for an additional 15–20 min. [*P_d*(hexanol) could not be reliably determined during this period because of the large number of ¹⁴C counts that had entered the serosal bath during the basal period.] Maximum stable flow rates were generally attained within 5 min. In some experiments, the basal and fully stimulated periods were separated by a period of submaximal stimulation with either vasopressin or 8-bromo cyclic AMP, as described in the following paper (Levine et al., 1984). *P_d*(w) and *P_d*(hexanol) were calculated from Eq. 1, and *P_f* was calculated from the defining equation:

$$P_f = \frac{J_v}{\bar{V}A\Delta c} \quad (2)$$

where *J_v* = volume flow of water; \bar{V} = partial molar volume of water (= 18 cm³/mol); *A* = area of membrane; Δc = concentration difference of solute (176 mosmol) in the two compartments.

When amphotericin B was used to assess series barrier permeabilities, basal permeabilities were first determined as described above. Both compartments were then drained, and the mucosal bath was replaced by fresh, dilute Ringer's solution containing, in addition to THO and [¹⁴C]hexanol, amphotericin B (purchased as Fungizone from Squibb, Princeton, NJ) at a concentration of 44 μg/ml; the serosal bath was replaced by fresh, undiluted Ringer's solution. After 45 min, by which time the amphotericin B effect was maximal, the serosal bath was removed² and replaced with fresh Ringer's solution. Stirring was then begun, with sampling and flow measurements performed over the next 10–15 min as previously described. Sodium desoxycholate, present in Fungizone in equal weight with amphotericin B, was found in control experiments to have no effect on either tracer fluxes or osmotic water flow.

In some experiments, series barrier permeabilities were assessed using both vasopressin and amphotericin B. In those cases, the vasopressin determination was made first. It was then washed out of the system, and the tissue was allowed to return to a basal level. A new basal permeability was then determined, followed by amphotericin B treatment as described above.

At the end of every experiment, both compartments were washed out and the mucosal

² A sample was taken for counting to determine the number of counts that left the mucosal bath during the 45-min equilibration period. Subtracting this from the original number present gave us the amount in the mucosal bath at the beginning of the test period.

bath was refilled with undiluted Ringer's solution containing [^{14}C]sucrose; the serosal bath, as usual, was refilled with undiluted Ringer's solution. If $P_d(\text{sucrose})$ was $>10^{-5}$ cm/s, or water flow (in the absence of an osmotic gradient) exceeded 0.2 $\mu\text{l}/\text{min}$, the experiment was discarded, because of presumed tissue damage at some time during its course.

P_f 's were corrected for a hydraulic series barrier permeability of 418×10^{-4} cm/s (Levine and Kachadorian, 1981; Kachadorian and Levine, 1982). In calculating $P_d(w)$, the measured flux of THO was corrected for the amount moving with the bulk water flow by subtracting from the measured flux a quantity equal to the specific activity of THO in the mucosal bath times the bulk flow rate. Since the specific activity in the transported fluid cannot possibly exceed the specific activity in the mucosal bath, this is a worst-case correction; it was never greater than 15%.

RESULTS

Measurements on Millipore Filters

These experiments were designed to determine the thickness of unstirred aqueous layers in the transport chamber used for toad bladder experiments. The approach was to take a membrane of known water permeability and then to measure its permeability in the transport chamber under the same stirring conditions used in the toad bladder experiments. The thickness of the unstirred aqueous layers in the toad bladder experiments was then calculated from the difference between the measured and known values of $P_d(w)$.

CALIBRATION OF FILTER Our first task was to determine the "known" permeability of some membrane. This was done in the high-speed chamber, under much more vigorous stirring conditions than possible, without damage, in toad bladder experiments. We chose an ultra-thin millipore filter (mean pore diameter 0.2 μm ; porosity 79%; thickness 25 μm [manufacturer's specifications]) having a micrometer-measured thickness of 35 μm , and simultaneously measured $P_d(w)$ and $P_d(\text{hexanol})$,³ to obtain: $P_d(w) = 27.3 (\pm 1.3) \times 10^{-4}$ cm/s ($n = 4$). If the membrane were simply a layer of water of thickness $\delta(w)$, then

$$\delta(w) = \frac{D(w)}{P_d(w)} \quad (3)$$

where $D(w)$ is the self-diffusion constant of water (2.44×10^{-5} cm²/s). In this instance, $\delta(w) = 90 \mu\text{m}$. Thus, the water permeability of filter plus any unstirred layers is equivalent to that of a 90- μm -thick layer of water.

Unstirred layers in the high-speed chamber should be very small (Peterson and Gregor, 1959), and therefore the measured value of $P_d(w)$ for the ultra-thin filter should be close to its true value. To confirm this, we also measured in the

³ For membranes with a mean pore diameter of 0.2 μm , $P_d(w)/P_d(\text{hexanol})$ should equal 2.7, the ratio of the free diffusion constants of H₂O and hexanol in water. These millipore filters have such a high hydraulic conductivity, however, that the vigorous stirring might produce convective movement through them and thereby cause the measured values of $P_d(w)/P_d(\text{hexanol})$ to be considerably less than 2.7 (approaching 1 in the limit of convection-dominated fluxes). To ensure that the THO fluxes were not contaminated by convection, we simultaneously measured hexanol fluxes, and rejected experiments in which $P_d(w)/P_d(\text{hexanol})$ was <2.4 .

high-speed chamber $P_d(w)$ and $P_d(\text{hexanol})^3$ for a thicker millipore filter (micrometer-measured thickness = 130 μm) of the same mean pore diameter, density, and porosity as that of the ultra-thin filter. For this filter, $P_d(w) = 7.6 (\pm 0.3) \times 10^{-4}$ cm/s ($n = 3$), which is equivalent to $\delta(w) = 321 \mu\text{m}$. Plotting $\delta(w)$, the "effective thickness" of the filters, against δ , their measured thickness, and extrapolating to $\delta = 0$, we find that the unstirred layer thickness in the high-speed chamber is only 8 μm (Fig. 3). Subtracting this from 90 μm , the measured value of $\delta(w)$ for the ultra-thin filter, we obtain: $\delta(w) = 82 \mu\text{m}$, the true "effective thickness" of this filter, or $P_d(w) = 29 \times 10^{-4}$ cm/s, its true water permeability.

UNSTIRRED LAYER THICKNESS IN THE TRANSPORT CHAMBER We then measured $P_d(w)$ and $P_d(\text{hexanol})^3$ for the ultra-thin filter in the transport chamber under the same stirring conditions used in the toad bladder experiments, and obtained: $[P_d(w)]_{\text{measured}} = 19.1 (\pm 1.4) \times 10^{-4}$ cm/s ($n = 5$), or $[\delta(w)]_{\text{measured}} = 128$

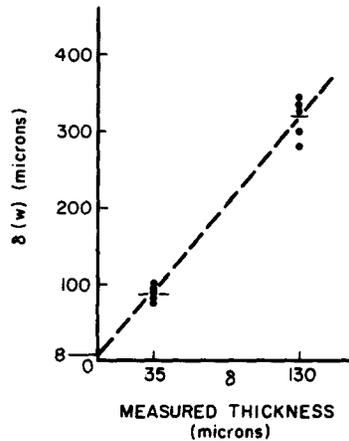


FIGURE 3. Relationship between measured thickness of the millipore filter (δ) and effective thickness $[\delta(w)]$. $\delta(w)$ is calculated as 2.44×10^{-5} cm²/s divided by $P_d(w)$.

μm . Since $[\delta(w)]_{\text{true}} = 82 \mu\text{m}$, we have for δ_{ul} , the unstirred layer thickness in the transport chamber:

$$\delta_{\text{ul}} = [\delta(w)]_{\text{measured}} - [\delta(w)]_{\text{true}} = 46 \mu\text{m}. \tag{4}$$

Thus, in the toad bladder experiments, the thickness of unstirred aqueous layers outside the tissue is 46 μm . [This is equivalent to $P_d(w) = 53 \times 10^{-4}$ cm/s and $P_d(\text{hexanol}) = 20 \times 10^{-4}$ cm/s.] This makes only a small correction (<15%) to measured values of $P_d(w)$ in toad bladder experiments (see below). In other words, the measured values of $P_d(w)$ reported below reflect intrinsic properties of fully stretched toad bladders and not properties of the chamber in which they were studied.

Determinations of $P_d(w)$ for Series Barriers within the Toad Bladder

The approach used was to so increase the water permeability of the luminal membrane that measured values of $\delta(w)$ [i.e., $P_d(w)$] essentially reflected the

characteristics of the series barriers within the tissue (plus the unstirred layers in the chamber). We employed two techniques to produce large water permeabilities in the luminal membrane: (a) treatment of the bladder's luminal surface with a high concentration of the polyene antibiotic amphotericin B, and (b) stimulation of the bladder with a maximal dose of ADH (vasopressin).

AMPHOTERICIN B EXPERIMENTS Amphotericin B is known to create aqueous pores in sterol-containing biological membranes and lipid bilayers (Cass and Dalmark, 1973; de Kruijff et al., 1974; Finkelstein and Holz, 1973). In particular, it has been shown to be effective on toad bladder, increasing its water permeability (Lichtenstein and Leaf, 1965). We observed large increases in P_f and $P_d(w)$ above basal levels in bladders exposed on their luminal surfaces to amphotericin B concentrations of 44 $\mu\text{g/ml}$ for 15–45 min. $P_d(w)$ increased from a basal value of $0.74 (\pm 0.04) \times 10^{-4}$ to $4.1 (\pm 0.2) \times 10^{-4}$ cm/s, and P_f increased from $2.4 (\pm 0.4) \times 10^{-4}$ to $71 (\pm 6) \times 10^{-4}$ cm/s ($n = 30$). Since $P_f/P_d(w) = 3$ for amphotericin B channels (as determined in lipid bilayer membranes [Holz and Finkelstein, 1970]),⁴ it is apparent from the much larger value of this ratio in these experiments that $P_d(w)$ is limited by series barrier constraints to THO diffusion; that is:

$$P_d^s(w) \approx [P_d^m(w)]_{\text{hi amph B}} \quad (5)$$

where the superscript "s" stands for series, and "m" stands for measured, as opposed to calculated, quantities.

$P_d^s(w)$ can be estimated directly from Eq. 5. In practice, however, it was calculated from the system of equations (see Fig. 4):

$$\frac{1}{[P_d^m(w)]_{\text{hi amph B}}} = \frac{1}{[P_d^{\text{lm}}(w)]_{\text{hi amph B}}} + \frac{1}{P_d^s(w)}; \quad (6)$$

$$[P_d^{\text{lm}}(w)]_{\text{hi amph B}} = [P_d^{\text{lm}}(w)]_{\text{basal}} + [P_d^{\text{lm}}(w)]_{\text{amph B channels}}; \quad (7)$$

$$\frac{1}{[P_d^m(w)]_{\text{basal}}} = \frac{1}{[P_d^{\text{lm}}(w)]_{\text{basal}}} + \frac{1}{P_d^s(w)}; \quad (8)$$

$$[P_d^{\text{lm}}(w)]_{\text{amph B channels}} = \frac{1}{3} (P_f^{\text{lm}})_{\text{amph B channels}}, \quad (9)^5$$

where the superscript "lm" stands for luminal membrane. The values of $P_d^s(w)$ calculated from Eqs. 6–9 were only ~25% larger than those obtained directly

⁴ In the following paper (Levine et al., 1984), we confirm this value in toad bladder.

⁵ $(P_f^{\text{lm}})_{\text{amph B channels}}$ was calculated from $(P_f^m)_{\text{hi amph B}}$ by correcting the latter quantity for a hydraulic series barrier (P_f^{sb}) of 418×10^{-4} cm/s (Levine and Kachadorian, 1981) using the equation:

$$\frac{1}{(P_f^m)_{\text{hi amph B}}} = \frac{1}{P_f^{\text{sb}}} + \frac{1}{(P_f^{\text{lm}})_{\text{hi amph B}}}$$

and then subtracting $(P_f^m)_{\text{basal}}$. This correction increases $(P_f^{\text{lm}})_{\text{hi amph B}}$ by ~15% (from 61 to 71 $\times 10^{-4}$ cm/s) and (from Eq. 9) increases $[P_d^{\text{lm}}(w)]_{\text{amph B channels}}$ to a similar extent (from 19.5 to 22.9 $\times 10^{-4}$ cm/s). Because both of these P_d values are so large compared with $[P_d^m(w)]_{\text{hi amph B}}$ (4.1×10^{-4} cm/s), the value of $P_d^s(w)$ calculated from Eq. 6 is virtually unaffected by the correction.

(and much more easily) from Eq. 5, which confirms that the luminal membrane contributed little to diffusional resistance to THO.

The result from the amphotericin B experiments was: $P_d^s(w) = 5.2 (\pm 0.2) \times 10^{-4}$ cm/s. This is equivalent to the permeability of a layer of water 469 μm in thickness. Since $P_d^s(w)$ includes the 46- μm unstirred layer outside the tissue (Eq. 4), we subtracted this from 469 μm to obtain:

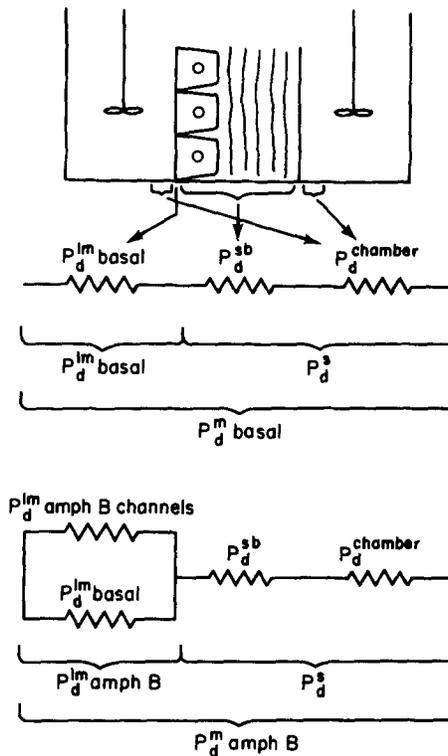


FIGURE 4. Schematic drawing of barriers to water diffusion in toad bladder in basal state and after stimulation by amphotericin B. Labeling is the same as in Eqs. 5-9.

$$\begin{aligned} \delta^{sb}(w) &= 423 \mu\text{m}; \\ P_d^{sb}(w) &= 5.8 \times 10^{-4} \text{ cm/s, (determined using amph B)} \end{aligned} \quad (10)$$

where the superscript "sb" stands for series barriers. Thus, the barriers to THO diffusion within the bladder, i.e., in series with the luminal membrane, have an effective thickness of 423 μm .

ADH EXPERIMENTS In these experiments, the bladder was stimulated with a maximum dose of vasopressin (20 mU/ml) to produce large increases in P_f and $P_d(w)$ above basal levels. $P_d(w)$ increased from $0.69 (\pm 0.03) \times 10^{-4}$ to $5.0 (\pm 0.1) \times 10^{-4}$ cm/s and P_f increased from $2.3 (\pm 0.4) \times 10^{-4}$ to $236 (\pm 11) \times 10^{-4}$ cm/s

($n = 35$). If we assume that measured values of $P_d(w)$ are limited by the series constraints to diffusion of water, then

$$P_d^s(w) \approx [P_d^m(w)]_{hiADH}. \quad (11)$$

$P_d^s(w)$ can be obtained directly from Eq. 11. Again, however, we made more accurate determinations using a system of equations analogous to that used with amphotericin B (see Levine et al., 1984). The values of $P_d^s(w)$ calculated by this more laborious procedure were only ~20% larger than those determined directly from Eq. 11.

The result from the ADH experiments was: $P_d^s(w) = 6.1 (\pm 0.2) \times 10^{-4}$ cm/s. This is equivalent to the permeability of a layer of water 400 μ m in thickness. Again, subtracting the 46- μ m unstirred layer outside the tissue, we obtain (Table I):

$$\begin{aligned} \delta^{sb}(w) &= 354 \mu\text{m}; \\ P_d^{sb}(w) &= 6.9 \times 10^{-4} \text{ cm/s. (determined using ADH)} \end{aligned} \quad (12)$$

TABLE I

Effective Thickness of Series Barriers within the Bladder to Water [$\delta^{sb}(w)$] and to Hexanol [$\delta(\text{hexanol})$]

Bladder treatment	$10^4 P_d^s(w)$ cm/s	$\delta^{sb}(w)$ μm	$10^4 P_d(\text{hexanol})$ cm/s	$\delta(\text{hexanol})$ μm
High amphotericin B	5.8 ± 0.2 ($n = 30$)	423	10.2 ± 0.4 ($n = 16$)	88
High ADH	6.9 ± 0.2 ($n = 36$)	354		
Basal			8.3 ± 0.3 ($n = 23$)	109

δ 's are related to their corresponding P_d 's through the equation:

$$\delta = \frac{D}{P_d}$$

where D is the diffusion constant for the molecule (2.44×10^{-5} cm²/s for H₂O and 9.0×10^{-6} cm²/s for hexanol).

There is very good agreement (to within 20%) between the magnitude of the series barrier to THO diffusion determined by increasing luminal membrane water permeability with amphotericin B (Eq. 10) and the magnitude determined by increasing the luminal membrane water permeability with a maximal dose of ADH (Eq. 12). This agreement, using two different agents, indicates that $P_d^{sb}(w)$ was not altered by our measurements.

In summary, the barriers to THO diffusion within the bladder, in series with the luminal membrane, are equivalent to an unstirred aqueous layer ~400 μ m in thickness.

Hexanol Permeability [$P_d(\text{hexanol})$] of Bladder

The primary objective of the research in this and the following paper was to determine the true value of $P_f/P_d(w)$ for the luminal membrane of ADH-stimulated bladders. To this end, the determination of the series resistance within the bladder to THO diffusion, as described in the preceding section, was essential. We also measured, however, the permeability coefficient for a lipophilic molecule, hexanol. Although this quantity is not needed for determining the true

value of $P_i/P_d(w)$, it provides an interesting insight into the nature of the series barriers within the bladder (see Discussion).

The result from measurements on basal and amphotericin B-treated bladders was: $P_d(\text{hexanol}) = 6.2 \pm 0.2 \times 10^{-4}$ cm/s ($n = 39$); approximately the same values were obtained under basal and stimulated conditions (Table I). This is equivalent to the permeability of a layer of water 145 μm in thickness. Subtracting the 46- μm unstirred layer outside the tissue, we obtain:

$$[\delta(\text{hexanol})]_{\text{toad bladder}} = 99 \mu\text{m}. \quad (13)$$

Thus, with respect to diffusion of a lipophilic molecule (hexanol), the toad bladder⁶ is equivalent to a 99- μm -thick unstirred aqueous layer.

DISCUSSION

The principal aim of the experiments reported in this paper was to measure the resistance to THO diffusion of the entire experimental system, toad bladder plus chamber, exclusive of the luminal membrane; this is required for determining the true value of $P_i/P_d(w)$ in ADH- and 8-bromo cyclic AMP-stimulated bladders (see Levine et al., 1984). We first showed, using a previously calibrated millipore filter, that the thickness of unstirred aqueous layers in the well-stirred transport chamber was only 46 μm . We then found that the resistance to THO diffusion of barriers within the bladder, in series with the luminal membrane, was equivalent to that of an unstirred aqueous layer of $\sim 400 \mu\text{m}$. This finding was based on experiments in which the permeability to water of the luminal membrane was increased to such an extent that resistance to THO diffusion resided elsewhere in the tissue. Luminal membrane water permeability was increased by either amphotericin B or ADH, and good agreement was found between these two independent means of determining the series barrier permeability to water.

We also measured the permeability to a lipophilic molecule, hexanol, and found that with respect to the diffusion of such a molecule, the toad bladder is equivalent to an unstirred aqueous layer of $\sim 100 \mu\text{m}$. This value is not inconsistent with our micrometer-measured thickness (on bladders glutaraldehyde-fixed in the chamber at the end of several experiments) of $\sim 25 \mu\text{m}$, considering that the 35- μm ultra-thin millipore filter, with a porosity of 79%, has a similar equivalent unstirred layer thickness for hexanol (and any other molecule small compared with its pore diameter). The series resistance within the bladder for water diffusion ($\sim 400 \mu\text{m}$) is four times greater than for hexanol diffusion ($\sim 100 \mu\text{m}$).

Holz and Finkelstein (1970) used butanol permeability measurements to correct measured values of $P_d(w)$ on lipid bilayer membranes. Their rationale was that, both theoretically and experimentally, a bilayer's permeability to a lipophilic molecule (e.g., butanol) is large compared with that of the unstirred aqueous layers on its two sides. Hence, the unstirred-layer thickness, δ_{ul} , is directly obtained from the measured value of $P_d(\text{butanol})$ and can be used to correct $P_d(w)$ measurements on lipid bilayers. This same procedure was subsequently used uncritically to correct $P_d(w)$ measurements on ADH-stimulated cortical

⁶ $\delta^{ab}(\text{hexanol})$ includes the luminal membrane, and therefore it is designated as $[\delta(\text{hexanol})]_{\text{toad bladder}}$ in Eq. 13.

collecting tubules (Schafer and Andreoli, 1972). We believe, however, that it is inappropriate to use a lipophilic molecule to measure the series barrier resistance to water movement across a tissue, such as cortical collecting tubule or toad bladder, because the resistance offered to a lipophilic molecule is not the same as that offered to water. In particular, whereas post-luminal membranes (e.g., intracytosolic membranes, basolateral membrane) are negligible barriers for a lipophilic molecule, they are substantial barriers for water; in fact, the most likely path for water diffusion within a granular epithelial cell is around, rather than across, intracytosolic membranes. Our finding that the series barrier resistance for lipophilic molecule diffusion is fourfold less than for water diffusion justifies our belief that series barrier corrections to $P_d(w)$ cannot be based on tissue permeability to lipophilic molecules.

The large difference between $\delta^{sb}(w)$ ($\sim 400 \mu\text{m}$) and $\delta^{sb}(\text{hexanol})$ ($\sim 100 \mu\text{m}$) provides an insight into the nature of the series barrier resistance for water. It clearly implies involvement of a lipophilic structure(s) (bilayer[s]), since a hydrophilic porous structure, such as the basement membrane, is, if anything, more of a barrier to the large hexanol molecule than to the small water molecule. It is unlikely, moreover, that the subepithelial layers (e.g., connective tissue, muscle, mesothelium) significantly hinder water diffusion; indeed, our measurements of $P_d(w)$ in the high-speed chamber on tissues stripped of the epithelial layer indicate that these supporting layers contribute only $65 \mu\text{m}$ to $\delta^{sb}(w)$ (preliminary results; with less vigorous stirring, Hays and Franki [1970] obtained a larger effective thickness for the supporting layers). This then leaves the epithelial layer itself as the major contributor to the series barrier for water.

The two lipophilic candidates for series barrier in the granular epithelial cell are the basolateral membrane and intracytoplasmic membranes. It is doubtful that the basolateral membrane makes a large contribution to $\delta^{sb}(w)$. If it did, we would be forced to the unlikely assumption that it contained huge pores (radii $\sim 35 \text{ \AA}$), to explain measured values for $P_t/P_d(w)$ of >100 in bladders maximally stimulated with ADH. It may well be, therefore, that the cytoplasm of granular cells is so densely packed with membrane-enclosed organelles (granules?) that they offer a serious obstruction to water diffusion (although not, of course, to water flow around them or hexanol diffusion through them) through the cell. Alternatively, the basal cells, which cover most of the basement membrane, may permit unimpeded entry and passage of hexanol exiting the granular cell, while restricting water transport to the narrow spaces that separate basal and granular cells.

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REFERENCES

- Carvounis, C. P., N. Franki, S. D. Levine, and R. M. Hays. 1979. Membrane pathways for water and solutes in toad bladder. I. Independent activation of water and urea transport. *J. Membr. Biol.* 49:253-268.

- Cass, A., and M. Dalmark. 1973. Equilibrium dialysis of ions in nystatin-treated red cells. *Nature (Lond.)* 244:47-49.
- de Kruijff, B., W. J. Gerritsen, A. Oerlemans, R. A. Demel, and L. L. M. van Deenen. 1974. Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. *Biochim. Biophys. Acta.* 339:30-43.
- Di Bona, D. R., M. M. Civan, and A. Leaf. 1969. The cellular specificity of the effect of vasopressin on toad urinary bladder. *J. Membr. Biol.* 1:79-91.
- Finkelstein, A. 1976. Nature of the water permeability increase induced by antidiuretic hormone (ADH) in toad urinary bladder and related tissues. *J. Gen. Physiol.* 68:137-143.
- Finkelstein, A., and R. Holz. 1973. Aqueous pores created in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. In *Membranes, A Series of Advances*. G. Eisenman, editor. Vol. 2: Lipid Bilayers and Antibiotics. Marcel Dekker, Inc., New York. 377-408.
- Finkelstein, A., and P. A. Rosenberg. 1979. Single-file transport: implications for ion and water movement through gramicidin A channels. In *Membrane Transport Processes*. C. F. Stevens and R. W. Tsien, editors. Raven Press, New York. 3:73-88.
- Grantham, J. J., and M. B. Burg. 1966. Effect of vasopressin and cyclic AMP on permeability of isolated collecting tubules. *Am. J. Physiol.* 211:255-259.
- Handler, J. S., and J. Orloff. 1973. The mechanism of action of antidiuretic hormone. In *Handbook of Physiology: Renal Physiology*. S. R. Geiger, J. Orloff, and R. W. Berliner, editors. The Williams and Wilkins Co., Baltimore. 791-814.
- Hays, R. M., and N. Franki. 1970. The role of water diffusion in the action of vasopressin. *J. Membr. Biol.* 2:263-276.
- Hebert, S. C., and T. E. Andreoli. 1980. Interactions of temperature and ADH on transport processes in cortical collecting tubules. *Am. J. Physiol.* 238:F470-F480.
- Holz, R., and A. Finkelstein. 1970. The water and nonelectrolyte permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *J. Gen. Physiol.* 56:125-145.
- Kachadorian, W. A., and S. D. Levine. 1982. The effect of distension on ADH-induced osmotic water flow in toad urinary bladder. *J. Membr. Biol.* 64:181-186.
- Levine, S. D., M. Jacoby, and A. Finkelstein. 1984. The water permeability of toad urinary bladder. II. The value of $P_f/P_d(w)$ for the antidiuretic hormone-induced water permeation pathway. *J. Gen. Physiol.* 83:543-561.
- Levine, S. D., and W. A. Kachadorian. 1981. Barriers to water flow in vasopressin-treated toad urinary bladder. *J. Membr. Biol.* 61:135-139.
- Lichtenstein, N. S., and A. Leaf. 1965. Effect of amphotericin B on the permeability of the toad bladder. *J. Clin. Invest.* 44:1328-1342.
- Muller, J., W. A. Kachadorian, and V. A. DiScala. 1980. Evidence that ADH-stimulated intramembrane particle aggregates are transferred from cytoplasmic to luminal membranes in toad bladder epithelial cells. *J. Cell Biol.* 85:83-95.
- Peterson, M. A., and H. P. Gregor. 1959. Diffusion-exchange of exchange ions and nonexchange electrolyte in ion-exchange membrane systems. *J. Electrochem. Soc.* 106:1051-1061.
- Schafer, J. A., and T. E. Andreoli. 1972. Cellular constraints to diffusion: the effect of antidiuretic hormone on water flows in isolated mammalian collecting tubules. *J. Clin. Invest.* 51:1264-1278.
- Wade, J. B. 1980. Hormonal modulation of epithelial structures. *Curr. Top. Membr. Transp.* 13:123-147.