The Water Permeability of Toad Urinary Bladder

II. The Value of $P_f/P_d(w)$ for the Antidiuretic Hormone-induced Water Permeation Pathway

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ABSTRACT Using the methods described in the preceding paper (Levine et al., 1984) for measuring the magnitude of the water-permeable barriers in series with the luminal membrane, we correct measured values of $P_d(w)$ in bladders stimulated with low doses of antidiuretic hormone (ADH) or 8-bromo cyclic AMP to obtain their true values in the luminal membrane. Simultaneously, we also determine P_f. We thus are able to calculate $P_f/P_d(w)$ for the hormone-induced water permeation pathway in the luminal membrane. Our finding is that $P_f/P_d(w) \approx 17$. Two channel models consistent both with this value and the impermeability of the ADH-induced water permeation pathway to small nonelectrolytes are: (*a*) a long (≈50 Å), small-radius (≈2 Å) pore through which 17 water molecules pass in single-file array, and (*b*) a shower-head-like structure in which the stem is long and of large radius (≈20 Å) and the cap has numerous short, small-radius (≈2 Å) pores. A third possibility is that whereas the selective permeability to H_2O results from small-radius (≈2 Å) pores, the large value of $P_f/P_d(w)$ arises from their location in the walls of long tubular vesicles (~2 μm in length and 0.1 μm in diameter) that are functionally part of the luminal membrane after having fused with it. Aggregate-containing tubular vesicles of these dimensions have been reported to fuse with the luminal membrane in response to ADH stimulation and have been implicated in the ADH-induced hydroosmotic response.

INTRODUCTION

In the preceding paper (Levine et al., 1984), we described two methods for measuring $P_d^*(w)$, the diffusional water permeability coefficient of the barriers in series with the luminal membrane. Here, we apply those methods to correct the measured values of $P_d(w)$ in bladders stimulated with low doses of either antidi-

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uretic hormone (ADH) or 8-bromo cyclic AMP. This enables us to calculate $P_f/P_d(w)$ for the hormone-induced water permeation pathway in the luminal membrane. The value of this ratio has been sought for over 30 years. Before we detail our results, it is instructive to recall the history of both the experimental and theoretical attempts to determine this quantity.

Koefoed-Johnsen and Ussing (1953), in the first paper to deal with this issue, reported that ADH stimulation caused large increases in P_f but relatively modest increases in $P_d(w)$. This finding, originally made in frog skin, was subsequently confirmed in toad urinary bladder (Hays and Leaf, 1962). Since the value of $P_f/P_d(w)$ increases with pore radius for solvent movement through pores (e.g., Robbins and Mauro, 1960), it was concluded that water normally traversed the bladder (or skin) through pores, and that the action of ADH was either to increase their radius or to open new ones of larger radius (Koefoed-Johnson and Ussing, 1953; Hays and Leaf, 1962). Pore radii could in fact be calculated from the magnitudes of $P_f/P_d(w)$ (Andersen and Ussing, 1957), and indeed determinations of "equivalent pore radii" were made in several cells, tissues, and synthetic membranes (e.g., Prescott and Zeuthen, 1953; Paganelli and Solomon, 1957; Pappenheimer, 1953; Durbin, 1960).

Dainty (1963) pointed out, however, that unstirred aqueous layers generally cause much larger reductions in experimental determinations of $P_d(w)$ than of P_f and that, consequently, reported values of $P_f/P_d(w)$ in various systems were spuriously high. Hays and Franki (1970) subsequently demonstrated that the value of $P_f/P_d(w)$ declined dramatically with increased vigorous stirring in ADH-stimulated toad bladder. They obtained a minimum value of ~40, but recognized that barriers within the tissue, inaccessible to stirring, could be responsible for this rather high number and that the true value could be considerably smaller, and might even be 1. In sum, the value of $P_f/P_d(w)$ for ADH-induced water permeability might lie anywhere between 1 and 40.

At this juncture, results from permeability studies on lipid bilayer membranes had an important influence on the interpretation of experiments in ADH-responsive tissues. For lipid bilayers, $P_f/P_d(w) = 1$ (Cass and Finkelstein, 1967). Furthermore, the absolute values of the water permeability coefficient, which depend on the lipid composition of the bilayer, encompass virtually the entire range of values reported for plasma membranes (Finkelstein, 1972). It was therefore suggested that water traversed the luminal membrane through the bilayer proper, and hence the true value of $P_f/P_d(w)$ was 1; ADH-induced water permeability was a consequence of an increased fluidity of the bilayer resulting from modifications of its composition or structure (Schafer et al., 1974; Pietras and Wright, 1975). Finkelstein (1976*b*) pointed out, however, that this picture was not physically consistent with the negligible increases in permeability to most nonelectrolytes after ADH stimulation (Pietras and Wright, 1975), for in lipid bilayers, modifications of bilayer composition and structure that increased water permeability resulted in comparable increases in nonelectrolyte permeabilities (Finkelstein, 1976*a*). Finkelstein concluded that ADH-induced water movement proceeded through pores. Since these pores let through water but not larger molecules (not even urea) (Grantham and Burg, 1966; Levine et al., 1973), they

had to be very narrow—at least at some point in their length. Because of the known monotonic decrease in $P_f/P_d(w)$ with decreasing pore radius, it was generally assumed that $P_f/P_d(w)$ for such narrow channels was close to 1.

Once again, however, work from lipid bilayer membranes influenced the interpretation of experiments. Levitt (1974) had argued theoretically that for a channel so narrow that water molecules cannot pass each other within it, $P_f/P_d(w) = N$, where N is the number of water molecules in single-file array. Rosenberg and Finkelstein (1978) then found this theoretical expectation realized for gramicidin A channels in lipid bilayer membranes. Since the inferred radius of the ADH-induced channel implied single-file transport there as well, Finkelstein and Rosenberg (1979) suggested that a correct determination of $P_f/P_d(w)$ would reveal the number of water molecules in that channel, i.e., the number in the narrow region of the channel where single-file transport occurred.

In summary, if series barrier contributions to measured values of $P_d(w)$ were minimized and corrected for, a proper determination of $P_f/P_d(w)$ in ADH-

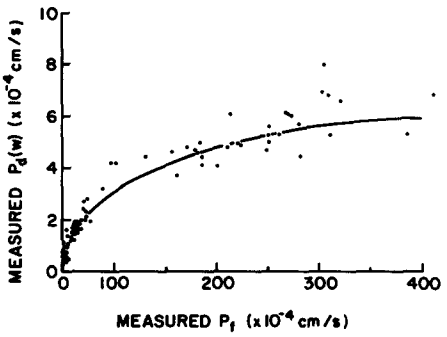

FIGURE 1. Measured values of P_f and $P_d(w)$ in the basal state and after stimulation by varying concentrations of ADH or 8-bromo cyclic AMP.

stimulated bladders could be made and its value related to channel structure. We claim to have made such a proper determination through the experiments presented in this paper. We report that $P_f/P_d(w) \approx 17$ for the ADH-induced (or 8-bromo cyclic AMP-induced) water permeation pathway, and we discuss possible structures consistent with this and other data on this pathway.

MATERIALS AND METHODS

General Considerations

Because of series barriers to THO diffusion within the bladder, the plot of measured values of P_f vs. measured values of $P_d(w)$ [i.e., P_f^m vs. $P_d^m(w)$] is nonlinear in ADH-stimulated tissues (Fig. 1). To determine true values of $P_d(w)$, and hence the correct value of $P_f/P_d(w)$, we simultaneously measured P_f and $P_d(w)$ under three successive conditions in each tissue: (*a*) in the basal state, (*b*) at low levels of stimulation, where the luminal membrane rather than the series barriers offers the major resistance to THO diffusion, and (*c*) at maximal levels of stimulation, where $P_d^m(w) \approx P_d^s(w)$, as discussed in the preceding paper (Levine et al., 1984). Calculations were based on the equations for permeabilities in series

and in parallel (Fig. 2). In each experiment, $P^s_d(w)$, the series permeability to water diffusion, was determined by treating the bladder with either a large dose of amphotericin B, a maximal dose of ADH, or both, as previously described (Levine et al., 1984), and the three unknowns pertaining to the luminal membrane (lm)—$[P_d(w)]_{basal}$, $[P_d(w)]_{low\ stim}$, $[P_d(w)]_{hi\ stim}$—were derived from the corresponding measured quantities (see Appendix A).

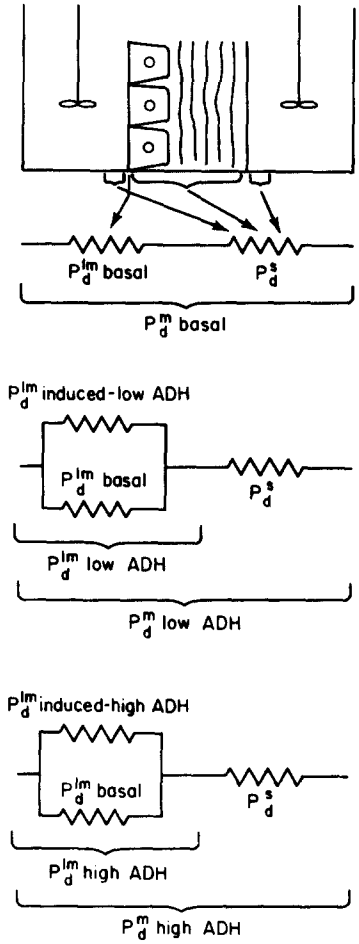

FIGURE 2. Schematic drawing of barriers to water diffusion in toad bladder in the basal state and during stimulation with low (0.5–0.75 mU/ml) and maximally stimulating (20 mU/ml) levels of ADH. Labeling as in Appendix Eqs. A1–A4 and A1′–A4′.

Experiments were performed in the transport chamber in a manner similar to that described by Levine et al. (1984). The serosal bath was filled with amphibian phosphate Ringer's solution (220 mosmol); the mucosal bath was filled with dilute Ringer's solution (one part Ringer's solution plus four parts distilled water) containing THO and [^{14}C]-hexanol at concentrations 1 and 0.1 μCi/ml, respectively. In some experiments, ampho-

tericin B was added to the mucosal bath at a final concentration of 2.5–5 (low concentration) or 45 μg/ml (high concentration). Water flow measurements and isotope flux measurements were made as described by Levine et al. (1984). In each experiment, the "low-stimulation" data were obtained in the 8–16-min period during which P_f and $P_d(w)$ were most nearly constant, whereas the "high-stimulation" data were obtained in the 8–16-min period during which P_f and $P_d(w)$ were maximal. At the end of every experiment, mucosal and serosal baths were drained and refilled with undiluted Ringer's solution, with the former also containing [^{14}C]sucrose. Experiments were discarded if either P_d(sucrose) was $>10^{-5}$ cm/s or water flow in this "gradient-free" state exceeded 0.2 μl/min.

Individual Experimental Protocols

EXPERIMENTS TO DETERMINE $P_f/P_d(w)$ IN ADH-STIMULATED AND 8-BROMO CYCLIC AMP-STIMULATED TISSUES After a 15-min basal period, vasopressin (0.5–0.75 mU/ml) or 8-bromo cyclic AMP (30 μM) was added to the serosal bath, beginning a low-stimulation period of 30 min. The serosal bath was then removed and replaced with fresh Ringer's solution containing 20 mU/ml vasopressin, and sampling was continued during the 30-min high-stimulation period. In some experiments a second high-stimulation period was obtained as follows: the serosal bath was replaced with vasopressin-free Ringer's solution and a new basal state was established. Both mucosal and serosal baths were then drained; the former was replaced with dilute Ringer's solution containing 45 μg/ml amphotericin B (plus 1 μCi/ml THO), and the latter was replaced with undiluted Ringer's solution. Sampling and flow measurements were made during the subsequent 30-min period.

EXPERIMENTS TO DETERMINE $P_f/P_d(w)$ IN AMPHOTERICIN B-STIMULATED TISSUES

(a) Low, followed by high, concentrations of amphotericin B. After a 15-min basal period, the mucosal bath was replaced with isotope-containing dilute Ringer's solution having 2.5–5 μg/ml amphotericin B, and the serosal bath was replaced with fresh, full-strength Ringer's solution. Measurements were made during this 30-min low-stimulation period. The baths were then replaced again, this time with the mucosal bath containing 45 μg/ml amphotericin B. Measurements were made for an additional 30 min during this high-stimulation period.

(b) High concentrations of vasopressin followed by low concentrations of amphotericin B. Since bladders exposed to amphotericin B responded poorly to subsequent vasopressin stimulation, bladders were first stimulated with 20 mU/ml vasopressin, following a 15-min basal period. After data were taken for 30 min during this high-stimulation period, the serosal bath was replaced several times until a new basal period was established. Following this, both mucosal and serosal baths were changed as described above, with the former containing 2.5–5 μg/ml amphotericin B. Data were then taken for 30 min during this low-stimulation period.

We deliberately worked within a restricted range of $P_d(w)$ values induced by low concentrations of ADH and 8-bromo cyclic AMP. We only accepted those experiments in which:

$$[P_d(w)]_{\text{measured with low stim}} > 1.5[P_d(w)]_{\text{basal}}$$

and

$$[P_d(w)]_{\text{measured with low stim}} < \tfrac{1}{2}P^s_d(w).$$

The first condition means that our values for stimulus-induced $P_d(w)$ were not critically dependent on basal values. The second condition means that measured values of $P_d(w)$ were never corrected by more than a factor of 2 for series barrier contributions, and

TABLE I

Measurements of P_f and $P_d(w)$ in Low-Dose 8-Bromo Cyclic AMP-stimulated Bladders

	Measured values*				Calculated values			
	Basal		Stimulated			Luminal membrane		
	P_f	$P_d(w)$	P_f	$P_d(w)$	$P^s_d(w)$	$P_f$ind‡	$P_d(w)$ind	$P_f$ind/$P_d(w)$ind
	2.5	1.1	29.8	1.9	5.5	27.4	1.6	17.0§ (16.9)
	3.7	0.8	29.8	1.8	6.1	26.1	1.5	17.0
	5.0	0.5	32.5	1.5	5.0	27.5	1.6	16.8 (17.1)
	7.6	1.1	23.5	1.7	5.0	16.0	1.2	13.9 (13.3)
	1.2	0.8	11.9	1.4	7.4	10.7	0.8	12.8 (11.6)
	5.0	0.5	27.2	1.7	4.6	22.2	2.0	11.1
	1.2	0.6	95.9	3.2	7.3	94.7	5.1	18.6
	0.0	0.9	28.1	1.8	5.5	28.1	1.6	17.7
	0.0	0.6	28.3	1.7	7.1	28.3	1.5	19.2
	0.0	0.8	29.9	1.9	5.5	29.9	2.0	15.2
	1.2	0.6	38.0	1.8	5.9	36.7	2.1	17.9
$\bar{X}$	2.5	0.8	34.1	1.9	5.9	31.6	1.9	16.1
SEM	0.8	0.1	6.5	0.3	0.3	6.7	0.3	0.8

* All permeabilities are $\times 10^{-4}$ cm/s. Measured P_f values are corrected for series flow barrier of $P_f = 418 \times 10^{-4}$ cm/s. Measured P_d values are corrected for isotope movement attributable to bulk water flow.

‡ ind = induced in luminal membrane by low concentrations of 8-bromo cyclic AMP.

§ P_f and $P_d(w)$ (stimulated) were obtained using "low" stimulation with 8-bromo cyclic AMP; $P^s_d(w)$ was determined using "high" stimulation with ADH. In calculating $P^s_d(w)$ and $P_d(w)$ind, we assumed that $P_f/P_d(w)$ for 8-bromo cyclic AMP stimulation = $P_f/P_d(w)$ for ADH stimulation. Data in parentheses are for experiments where, in addition, amphotericin B was used for high stimulation (i.e., for determining $P^s_d(w)$. We assumed that $P_f/P_d(w) = 4$ for amphotericin B channels.

TABLE II

Measurements of P_f and $P_d(w)$ in Low-Dose ADH-stimulated Bladders

	Measured values*				Calculated values			
	Basal		Stimulated			Luminal membrane		
	P_f	$P_d(w)$	P_f	$P_d(w)$	$P^s_d(w)$	$P_f$ind‡	$P_d(w)$ind	$P_f$ind/$P_d(w)$ind
	0.0	0.5	50.8	2.4	7.9	50.8	2.8	18.3§ (15.7)
	2.5	0.9	44.7	2.5	6.9	42.2	2.9	14.5 (12.3)
	3.7	0.7	28.1	1.8	5.8	24.3	1.9	12.9 (11.2)
	0.6	0.5	61.2	2.0	6.8	60.6	2.2	27.1 (22.4)
	5.0	0.6	28.5	1.5	6.1	23.5	1.4	16.9 (15.5)
	1.2	0.8	38.0	1.6	5.8	36.7	1.3	28.2 (20.2)
	3.7	0.8	19.4	1.3	9.1	15.7	0.6	24.6 (22.6)
	3.7	0.6	46.4	2.0	6.1	42.7	2.3	18.7 (15.1)
	0.0	0.6	52.2	2.1	6.5	52.2	2.5	21.2 (18.5)
$\bar{X}$	2.3	0.7	41.0	1.9	6.8	38.7	2.0	20.3 (17.1)
SEM	0.6	0.1	4.5	0.1	0.4	5.0	0.3	1.8 (1.4)

* Permeabilities as in Table I.

‡ ind = induced in luminal membrane by low concentrations of ADH.

§ Experiments were performed using ADH for both low and high stimulation. Data in parentheses are for experiments where, in addition, amphotericin B was used for high stimulation as in Table I.

therefore our calculated values of $P_d(w)$ for the luminal membrane were not critically dependent on the values of $P_d^s(w)$.

RESULTS

Tables I and II summarize the results of our determinations of stimulus-induced P_f and $P_d(w)$ in bladders treated with low doses of either 8-bromo cyclic AMP (Table I) or ADH (Table II); Fig. 3 plots stimulus-induced P_f^{lm} vs. stimulus-induced $P_d^{lm}(w)$ as given in the tables. The first five columns of the tables give the measured values of P_f and $P_d(w)$ (for basal and stimulated conditions) and the values of $P_d^s(w)$; the next two columns give the calculated values for P_f^{lm} and

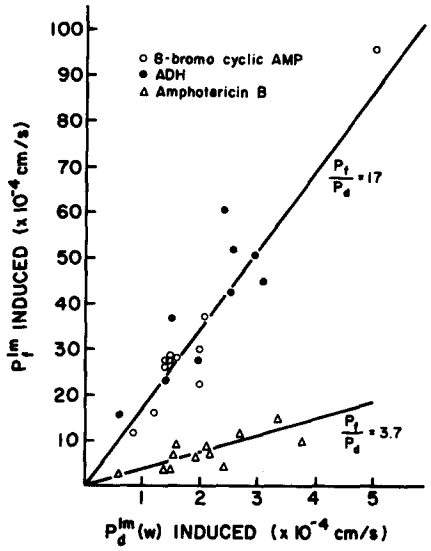

FIGURE 3. Relationship between stimulus-induced $P_d^{lm}(w)$ and stimulus-induced P_f^{lm} in bladders receiving low concentrations of 8-bromo cyclic AMP (○), ADH (●), or amphotericin B (△). Where both "high" amphotericin and "high" ADH were used for determination of $P_d^s(w)$, data shown are the means of the two resulting values for $P_d^{lm}(w)$ induced. Data are from Tables I–III.

$P_d^{lm}(w)$ induced in the luminal membrane by the stimulus; and the last column gives the ratio of these two quantities. The calculations were performed as described by Levine et al. (1984) and in Appendix A.

Two points are noteworthy about the calculations: first, the correction to P_f from the hydrodynamic series barrier (Levine and Kachadorian, 1981) is small (≈10%); that is, the calculated values for the stimulus-induced P_f in the luminal membrane could just as well have been obtained by subtracting the basal values of P_f from the values of P_f in stimulated bladders, without applying any series barrier corrections. Second, the corrections to $P_d(w)$ from the diffusional series barrier, although significant compared with those for P_f, are less than a factor of 2. This resulted from a judicious choice of low concentrations of ADH and 8-bromo cyclic AMP to give submaximal stimulations; that is, the water permea-

bility of the luminal membrane did not become so large that the measured values of $P_d(w)$ were dominated by the series barriers.

The average values for $P_f/P_d(w)$ of the water permeation pathway induced in the luminal membrane by 8-bromo cyclic AMP and ADH were 16.1 ± 0.8 (11 experiments) and 18.7 ± 1.6 (9 experiments), respectively; the difference is not experimentally significant. The above values were obtained using either the ADH-determined or the amphotericin B-determined series barrier corrections, and were not dependent on which correction was applied.

By all rational criteria we have dealt appropriately with the series barrier problem, and therefore our calculated value of ≈17 for $P_f/P_d(w)$ of the ADH-induced (or 8-bromo cyclic AMP-induced) water permeation pathway in the luminal membrane is correct. Given the complexity of the tissue and the assumptions underlying our calculations,[1] however, it is highly desirable to have an independent test of our methodology. This is provided by our determination of

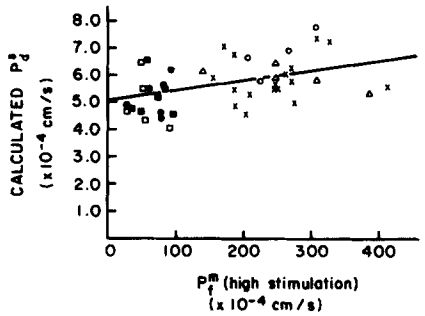

FIGURE 4. Relationship between high-stimulated P_f and $P_d^s(w)$, calculated from bladders receiving: low 8-bromo cyclic AMP and high ADH (×); low 8-bromo cyclic AMP and high amphotericin B (■); low ADH and high ADH (○); low ADH and high amphotericin B (●); low amphotericin B and high ADH (△); low amphotericin B and high amphotericin B (□).

$P_f/P_d(w)$ for the amphotericin B-induced channels in the luminal membrane. The average value for $P_f/P_d(w)$ of these channels was 3.7 ± 0.5 (12 experiments) (Table III and Fig. 3).

There are two significant points about this value *vis-à-vis* our result for the ADH-induced (or 8-bromo cyclic AMP-induced) water permeability. First, it demonstrates our ability to obtain relatively low values of $P_f/P_d(w)$ (in this case, 3.7) if they occur. Note in Fig. 3 that the amphotericin B and the ADH (8-bromo cyclic AMP) experiments cover the same range of values for $P_d(w)$, but the values for P_f are much smaller in the former. Thus, our determinations of $P_d(w)$ do not intrinsically result in large values for $P_f/P_d(w)$. Second, the value of 3.7 as

[1] One of the most worrisome assumptions is that $P_d^s(w)$ of a given bladder is the same at low levels of stimulation with ADH, 8-bromo cyclic AMP, or amphotericin B (where we apply it as a correction) as at high levels of stimulation (where we measure it). Clearly, we cannot determine $P_d^s(w)$ at low levels of stimulation. Nonetheless, we found that there was only an ~30% variation in $P_d^s(w)$ over a 10-fold range of high-stimulated values of P_f^m (see Fig. 4).

determined on the toad bladder is in good agreement with the value of 3.0 as determined on lipid bilayer membranes (Holz and Finkelstein, 1970).[2] Since our methodology gives the "right" result for the amphotericin B channel, it should also give the right result for the ADH-induced (or 8-bromo cyclic AMP-induced) water permeability.

TABLE III

Measurements of P_f and $P_d(w)$ in Low-Dose Amphotericin B-stimulated Bladders

	Measured values*				Calculated values‡			
	Basal		Stimulated			Luminal membrane		
	P_f	P_d(w)	P_f	P_d(w)	P^s_d(w)	$P_f$ind§	P_d(w)ind	$P_f$ind/P_d(w)ind
	2.5	0.8	5.9	1.7	6.9	3.4	1.4	2.4
	0.9	0.7	10.5	1.8	7.4	9.5	1.6	5.9
	3.7	0.7	14.4	2.2	4.1	10.7	3.8	2.8
	5.0	1.3	9.5	2.2	4.6	4.5	2.5	1.8
	5.0	0.9	13.0	2.2	6.5	8.0	2.2	3.6
	1.2	0.5	8.3	1.5	5.5	7.0	1.6	4.5
	6.3	0.8	18.4	2.0	4.3	12.1	2.7	4.5
	3.7	0.5	7.4	1.5	6.2	3.7	1.5	2.5
	4.7	0.7	13.8	2.0	6.0	9.1	2.2	4.2
	2.5	0.5	5.3	1.0	6.5	2.8	0.6	4.6
	9.0	0.5	15.7	1.8	5.8	6.6	2.0	3.3
	4.4	1.0	19.4	2.5	5.4	15.1	3.4	4.5
$\bar{X}$	4.1	0.7	11.8	1.9	5.8	7.7	2.1	3.7
SEM	0.6	0.1	1.4	0.1	0.3	1.1	0.3	0.3

* Permeabilities as in Table I.

‡ The first seven experiments used high concentrations of amphotericin B for determination of P^s_d(w). The remainder used high concentrations of ADH, assuming P_f/P_d(w) = 20 for ADH-induced luminal membrane permeability.

§ ind = induced in luminal membrane by low concentrations of amphotericin B.

DISCUSSION

The Value of $P_f/P_d(w)$

The basic conclusion from the experiments reported in this and the preceding paper is that P_f/P_d(w) is large ($\approx$17) for the water permeation pathway induced in the luminal membrane of toad urinary bladder by ADH. Recently, a value of 9 has been reported for frog urinary bladder (Parisi and Bourguet, 1983); the value for cortical collecting tubules of mammalian kidneys is unclear.[3] In the

[2] The value of 3.0 was determined for "two-sided" amphotericin B channels. All nonelectrolyte permeability data indicate, however, that "one-sided" channels (which are the ones induced in the toad bladder) have essentially the same radius as "two-sided" channels, and therefore, by inference, the same value of P_f/P_d(w) (Kleinberg, 1983).

[3] We feel that THO experiments in cortical collecting tubules (Schafer et al., 1974; Al-Zahid et al., 1977; Hebert and Andreoli, 1980) are so dominated by series barrier contributions that luminal membrane P_d(w) cannot be unequivocally determined.

Introduction we summarized the 30-year history of attempts to determine the value of ADH-induced $P_f/P_d(w)$. The major problem has been the determination of $P_d(w)$, uncontaminated by series barrier and unstirred layer resistances to diffusion of isotopic water. We believe that we have satisfactorily solved this problem through our analysis in the preceding paper (Levine et al., 1984) of series barriers within the toad bladder and our subsequent use in this paper of $P_d^s(w)$ to correct measured values of $P_d(w)$. This belief is supported by the following: when we applied to amphotericin B-induced channels the identical methodology and procedures used for ADH-induced water permeability, we obtained a value for $P_f/P_d(w)$ of amphotericin B channels in toad bladder that was essentially the same as that previously obtained for them in lipid bilayer membranes.

In summary, $P_f/P_d(w)$ of the ADH-induced water permeation pathway in the luminal membrane of toad urinary bladder is certainly not 1, a long-held possibility; it is instead a rather large number, ≈ 17.

The Meaning of $P_f/P_d(w) \approx 17$

There are at least four possible interpretations of this large value for $P_f/P_d(w)$. Three of them relate to the structure of the water-permeable channels. The fourth attributes the value of $P_f/P_d(w)$ to structural changes in the luminal membrane associated with ADH-induced water permeability and does not relate to channel structure.[4] We shall consider each of these interpretations in turn.

LARGE-RADIUS PORES If Poiseuille's equation for laminar flow through macroscopic cylindrical tubes applies to pores of molecular dimension,[5] then a value of 17 for $P_f/P_d(w)$ could be explained by pores of ~15 Å radius. This would be a reasonable interpretation, if nothing else were known about ADH-induced water-permeable channels. In addition, however, these channels are impermeable to small nonelectrolytes such as acetamide and urea (Grantham and Burg, 1966; Levine et al., 1973; Carvounis et al., 1979). Barring hitherto unknown special forces that prevent small nonelectrolytes from entering or passing through them, the inescapable conclusion is that these channels have a very small radius of no more than 2 Å, at least somewhere in their length (see below). We therefore must reject the possibility that these channels are of large radius throughout their length.

SINGLE-FILE TRANSPORT For a channel so narrow that water molecules cannot pass one another, $P_f/P_d(w) = N$, where N is the number of water molecules in single-file array within the channel (Levitt, 1974). A possible interpretation of our result, therefore, is that ADH-induced water-permeable channels contain 17 water molecules that undergo single-file transport. A channel 50 Å in length and 2 Å in radius could contain 17 water molecules in single-file array; in

[4] In principle, water movement through any phase in which H_2O molecules "see" each other will give rise to values of $P_f/P_d(w)$ of >1. However, nonporous systems having the very large value of $P_f/P_d(w)$ that we report seem very unlikely to us.

[5] Although it is difficult to justify this assumption from first principles, it appears to be (surprisingly) valid, even for pores of only 2 Å radius (Finkelstein and Rosenberg, 1979).

addition, it would be impermeable to small nonelectrolytes such as acetamide and urea, and its length could span the lipid bilayer of the plasma membrane.

Other information, however, although not precluding this model, makes it somewhat doubtful. It is fairly certain that ADH-induced water permeability results from fusion with the luminal membrane of cytoplasmic tubular vesicles containing intramembranous particles arranged as "aggregates" (Kachadorian et al., 1977*a*; Muller et al., 1980; Wade, 1980); the water-permeable channels, originally present in the vesicles' membranes, insert into the luminal membrane. It is generally assumed, although not proven, that the channels are contained in the area occupied by the aggregates.[6] If they are, we can do some arithmetic: a fully stimulated bladder has $\sim 10^8$ aggregates/cm^2 (Kachadorian et al., 1977*b*) and a P_f of $\sim 5 \times 10^{-2}$ cm/s (Levine et al., 1984). The permeability of each aggregate is therefore:

$$(P_f)_{\text{aggregate}} = \frac{5 \times 10^{-2}}{10^8} = 5 \times 10^{-10} \text{ cm}^3/\text{s}. \quad (1)$$

The diameter of each aggregate is ~ 0.1 μm (Kachadorian et al., 1977*b*), and we must place there enough of the single-file channels to give this water permeability. From Poiseuille's Law, the hydraulic permeability of a channel 50 Å in length and 2 Å in radius is:

$$(p_f)_{\text{channel}} = 19 \times 10^{-15} \text{ cm}^3/\text{s} \quad \text{(predicted from Poiseuille's Law)}. \quad (2)$$

(This probably represents a maximum possible value for such a channel.) We must therefore pack 2.5×10^4 single-file channels of radius 2 Å into an area of $\sim 10^{-2}$ μm^2. The center-to-center distance between channels would be $\approx$6 Å, only slightly larger than their 4-Å internal diameter, thus requiring a wall thickness of only 2 Å. This does not seem compatible with any realistic molecular structure for the channels. (See also the "sieve" model suggested by Wade [1980], who makes a similar calculation to the one just given.)

SHORT, SINGLE-FILE REGION IN SERIES WITH A LONG, LARGE-RADIUS PORE

The third interpretation of the large value for $P_f/P_d(w)$ combines features of the previous two. Consider the model depicted in Fig. 5*A*. A right circular cylinder of radius R and length L has at its base a small hole of radius r and thickness l. Suppose that $R = 15$ Å and $r = 2$ Å. If p_f and $p_d(w)$ of the small, short hole were much larger than the corresponding water permeabilities of the cylinder in series with it, such a channel would display the major features of the ADH-induced, water-permeable channel; namely, impermeability to small nonelectrolytes such as urea, and $P_f/P_d(w) \approx 17$ (the value for the cylinder). It might appear that for a short enough hole, p_f and $p_d(w)$ would be large compared with their counter-

[6] The correlation between water permeability and number of aggregates is excellent (Kachadorian et al., 1975, 1977*a*; Levine and Kachadorian, 1981), but does not preclude the possibility that the aggregates are merely identifiable markers for channel-containing vesicles. That is, when such vesicles fuse with the luminal membrane, they perforce insert both channels and aggregates, but only the latter are distinguished in electron micrographs of freeze-fracture replicas.

parts in the series cylinder. This, however, is not the case. Even a hole of zero thickness has a finite hydrodynamic resistance.

Assuming macroscopic laws are still applicable for such a hole of molecular radius,[5] then

$$p_f = \frac{RT}{3\eta \bar{V}(w)} r^3, \tag{3}$$

where η is the viscosity of water, $\bar{V}(w)$ is its partial molar volume, and RT has its usual meaning (Happel and Brenner, 1975). For a 2-Å-radius hole at room temperature, this becomes

$$p_f = 4.1 \times 10^{-13} \text{ cm}^3/\text{s} \qquad (r = 2 \text{ Å}; l = 0) \tag{4a}$$

and because of single filing through this region, this will also be the value of $p_d(w)$:

$$p_d(w) = 4.1 \times 10^{-13} \text{ cm}^3/\text{s} \qquad (r = 2 \text{ Å}; l = 0). \tag{4b}$$

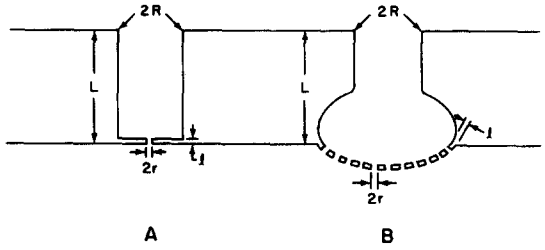

FIGURE 5. (*A*) Model of ADH-induced channel having a short, single-file region in series with a long, large-radius tube. (*B*) Model of ADH-induced channel having multiple, short, single-file regions in series with a long, large-radius tube.

On the other hand, if, for the large-radius cylinder in series with this hole, L = 50 Å (a reasonable length for a channel spanning the bilayer) and R = 15 Å, we have:

$$P'_f = 5.3 \times 10^{-11} \text{ cm}^3/\text{s} \tag{5a}$$

$$P'_d(w) = 2.9 \times 10^{-12} \text{ cm}^3/\text{s}. \qquad (R = 15 \text{ Å}; L = 50 \text{ Å}) \tag{5b}$$

Thus, the short, 2-Å-radius hole is rate limiting for both hydraulic and diffusional water movement, and consequently $P_f/P_d(w)$ for the channel is ~1.

We see from the above that although a long, wide cylinder with a short, 2-Å-radius hole in its base will be impermeable to urea, the value of $P_f/P_d(w)$ will be ~1, and not 17—the measured value for the ADH-stimulated bladder. On the other hand, if there were a large number of such holes in series with the long, large-radius cylinder, the latter would become rate limiting for water transport, and $P_f/P_d(w) \gg 1$. Geometrically, not enough holes can be placed in the base of the cylinder for this to occur, but if the base were expanded into a larger area, as in Fig. 5*B*, a sufficient number of holes could be accommodated.

For such a showerhead-like structure,

$$\left[\frac{P_f}{P_d(w)}\right]_{channel} = \frac{np_d(w) + P'_d(w)}{np_f + P'_f} \frac{P'_f}{P'_d(w)} \frac{p_f}{p_d(w)}, \quad (6)$$

where p_f and $p_d(w)$ pertain to the short, small-radius holes, n is their number, and P'_f and $P'_d(w)$ pertain to the long, fat stem.

As an example, if we arbitrarily take for the stem a length of 50 Å and a radius of 20 Å, and for the cap a circle of diameter 400 Å with 500 short (zero thickness) holes of 2 Å radius, then

$$p_f = p_d(w) = 4.1 \times 10^{-13} \text{ cm}^3/\text{s (zero thickness small holes of 2 Å radius)}$$

$$np_f = np_d(w) = 2.05 \times 10^{-10} \text{ cm}^3/\text{s} \qquad (n = 500)$$

$$\left.\begin{aligned} P'_f &= 1.93 \times 10^{-10} \text{ cm}^3/\text{s} \\ P'_d(w) &= 6.03 \times 10^{-12} \text{ cm}^3/\text{s} \end{aligned}\right\} \text{(stem of length 50 Å and radius 20 Å)}$$

and substituting these values into Eq. 6, we get:

$$\left[\frac{P_f}{P_d(w)}\right]_{channel} = 17.$$

Thus, this showerhead model of the ADH-induced channel holds back urea and has a value for $P_f/P_d(w)$ of 17. Moreover, six of these structures, which can be accommodated in an ADH-induced aggregate, have the water permeability attributed to such aggregates, namely, 5×10^{-10} cm^3/s (Eq. 1). Conceivably, the organization of an aggregate seen in freeze-fracture replicas reflects the morphology of showerhead channels.

TUBULAR VESICLE FUSION WITH THE LUMINAL MEMBRANE We pointed out earlier that ADH-induced water permeability apparently results from fusion with the luminal membrane of aggregate-containing tubular vesicles (see, for example, Wade, 1980). These tubular vesicles are ~2 μm in length and 0.1 μm in diameter (Kachadorian et al., 1981), and their membrane contains aggregates throughout the length of the tube (Wade, 1981). Some of these aggregates migrate out onto the surface of the luminal membrane, and because the ADH-induced water permeability is so well correlated with the number appearing on the luminal surface, it is generally assumed that the increased water permeability is through the region delineated by these surface aggregates. It is also possible, however, that the increased water permeability is through the entire tubular membrane, lined by aggregates and accessible to the luminal bath through the area of fusion (Muller et al., 1980). If this is the case, we can show (see Appendix B) that large values of $P_f/P_d(w)$ are obtainable even if $p_f/p_d(w) = 1$ for the individual water-permeable channels. In essence, the fusion of a long tubular vesicle with the luminal membrane introduces along with the water-permeable channels (which are now functionally part of the luminal membrane) its own series barrier to THO diffusion.

Concluding Remarks

Any model of the ADH-induced water permeation pathway must address two experimental facts: (*a*) the channels of this pathway, while permeable to H_2O, exclude most other nonelectrolytes, even those as small as urea; and (*b*) $P_f/P_d(w)$

≈ 17. We have presented two models for channel structure that are consistent with both of these facts. One is a long, narrow pore (radius ≈2 Å) with 17 water molecules in single-file array. The other is a showerhead-like object, in which the stem is long and of large radius (≈20 Å), and the cap has numerous short, small-radius pores (≈2 Å). The latter model is more consistent with morphological correlates of ADH-induced water permeability than is the former, but until a clearer interpretation of that morphology is available, the first model (the long, narrow pore) cannot be excluded. One feature of ADH-induced channels that we have not discussed is their low ion permeability (Finkelstein and Rosenberg, 1979). This is readily accounted for in a long, narrow pore from considerations of the Born energy for moving an ion into such a structure (Parsegian, 1969), but would probably require the proper placement of charge groups in the showerhead cap to curtail ion permeation through the short pores located there.

We have also considered a third model, in which the selective permeability to H_2O still results from small-radius (≈2 Å) pores, but the large value of $P_f/P_d(w)$ arises from their location in the walls of long tubular vesicles that are functionally part of the luminal membrane because of their fusion with it. If this model is correct, then the migration of aggregates out of the tubule onto the luminal surface is an epiphenomenon, at least insofar as water permeability is concerned, since most of the water transport occurs through pores (perhaps associated with aggregates) that are still in the walls of the tubule.[7] An attractive feature of this model is that there is an ~10-fold-larger area available for the pores than that indicated by the area occupied by the aggregates on the luminal surface, and therefore the contortions required to pack in enough channels to account for the osmotic water permeability of the fully stimulated tissue are avoided. In our opinion, this model is the one that explains the ADH-induced water permeability data most naturally.

APPENDIX A

The value of $P_d^s(w)$, the series permeability to THO, can be calculated from $[P_d^m(w)]_{hi\ ADH}$, the measured value of $P_d(w)$ in a bladder stimulated with a high concentration of ADH, by a system of equations analogous to Eqs. 6–9 used, in the preceding paper (Levine et al., 1984), with amphotericin B. Thus, we can write:

$$\frac{1}{[P_d^m(w)]_{hi\ ADH}} = \frac{1}{[P_d^{lm}(w)]_{hi\ ADH}} + \frac{1}{P_d^s(w)} \tag{A1}$$

$$[P_d^{lm}(w)]_{hi\ ADH} = [P_d^{lm}(w)]_{basal} + [P_d^{lm}(w)]_{induced\ by\ hi\ ADH} \tag{A2}$$

$$\frac{1}{[P_d^m(w)]_{basal}} = \frac{1}{[P_d^{lm}(w)]_{basal}} + \frac{1}{P_d^s(w)} \tag{A3}$$

but instead of Eq. 9 of the preceding paper, we have:

$$[P_d^{lm}(w)]_{induced\ by\ hi\ ADH} = \frac{1}{r}\,[P_f^{lm}]_{induced\ by\ hi\ ADH}, \tag{A4}$$

[7] Data on cytochalasin B-treated bladders are in apparent conflict with this model; namely, cytochalasin B has an inhibitory effect on both P_f and the number of aggregates appearing on the luminal surface (in ADH-stimulated bladders), but does not inhibit the number of fusion events (Muller et al., 1980).

where r is the ratio of P_f to $P_d(w)$ in the ADH-induced water permeation pathway. It is, of course, the quantity we ultimately wish to determine. In the experiments to determine this, we stimulated with submaximal levels of ADH (or 8-bromo cyclic AMP) and used the equations:

$$\frac{1}{[P_d^m(w)]_{low\ ADH}} = \frac{1}{[P_d^{lm}(w)]_{low\ ADH}} + \frac{1}{P_d^s(w)} \tag{A1'}$$

$$[P_d^{lm}(w)]_{low\ ADH} = [P_d^{lm}(w)]_{basal} + [P_d^{lm}(w)]_{induced\ by\ low\ ADH} \tag{A2'}$$

$$\frac{1}{[P_d^m(w)]_{basal}} = \frac{1}{[P_d^{lm}(w)]_{basal}} + \frac{1}{P_d^s(w)} \tag{A3'}$$

$$[P_d^{lm}(w)]_{induced\ by\ low\ ADH} = \frac{1}{r}[P_f^{lm}]_{induced\ by\ low\ ADH}. \tag{A4'}$$

Eqs. A1'–A4' can be solved simultaneously to find $P_d^s(w)$ and r. In practice, we assumed a value for r, calculated $P_d^s(w)$ from Eqs. A1–A4, substituted this into Eqs. A1'–A4' to get a new value of r, and then continued to iterate until we obtained self-consistent values for r and $P_d^s(w)$. In both high- and low-stimulated tissues, P_f^{lm} is calculated from the equation

$$\frac{1}{P_f^m} = \frac{1}{P_f^{lm}} + \frac{1}{P_f^s},$$

where $P_f^s = 418 \times 10^{-4}$ (cm/s) (Levine and Kachadorian, 1981).

APPENDIX B

Let us model a tubular vesicle fused to the luminal membrane as a right circular cylinder of radius R and length L (Fig. 6). Let the concentration of THO at the top (at $x = 0$) be maintained at c_o and its concentration everywhere else outside the cylinder be 0. The walls of the cylinder have a diffusion permeability coefficient for water given by P_d; for simplicity, we consider that the base of the cylinder is impermeable to water. (Note that P_d has the dimensions of centimeters per second.) In addition, we assume that L is much larger than R, so that there are no radial gradients of c, the THO concentration.

In the steady state, the continuity equation is:

$$-D\pi R^2 \frac{dc}{dx} = -\left(D\pi R^2 \frac{dc}{dx} + D\pi R^2 \frac{d^2c}{dx^2}\,dx\right) + 2\pi R P_d c\,dx,$$

which simplifies to

$$DR\frac{d^2c}{dx^2} - 2P_d c = 0. \tag{B1}$$

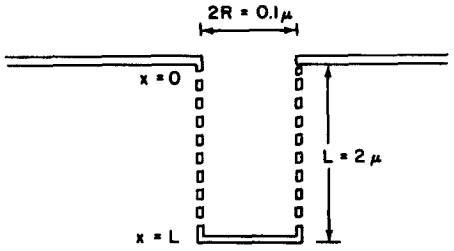

FIGURE 6. Model of tubular vesicle with water-permeable channels in its wall.

The solution of this equation is

$$c = c_1 e^{\alpha x} + c_2 e^{-\alpha x}, \tag{B2}$$

where α is defined as

$$\alpha \equiv \sqrt{\frac{2P_d}{DR}} \tag{B3}$$

and c_1 and c_2 are constants obtained from the boundary conditions. These boundary conditions are:

$$c = c_o \quad \text{at} \quad x = 0$$

$$\left(\frac{dc}{dx}\right)_{x=L} = 0.$$

Applying these conditions to Eq. B2 we get,

$$c_1 = c_o \frac{1}{1 + e^{2L\alpha}}$$

$$c_2 = c_o \frac{e^{2L\alpha}}{1 + e^{2L\alpha}}$$

and substituting these expressions into Eq. B2, we have:

$$c = \frac{c_o}{1 + e^{2L\alpha}} [e^{\alpha x} + e^{\alpha(2L-x)}]. \tag{B4}$$

The flux, $\Phi^*_{x=0}$, of THO into the cylinder at the top is:

$$\Phi^*_{x=0} = -D\pi R^2 \left(\frac{dc}{dx}\right)_{x=0}. \tag{B5}$$

From Eq. B4, we have:

$$\left(\frac{dc}{dx}\right)_{x=0} = \alpha c_o \frac{1 - e^{2L\alpha}}{1 + e^{2L\alpha}}$$

and substituting this into Eq. B5, we obtain

$$\Phi^*_{x=0} = -D\pi R^2 \alpha c_o \left(\frac{e^{-L\alpha} - e^{L\alpha}}{e^{-L\alpha} + e^{L\alpha}}\right). \tag{B6}$$

Eq. B6 is the expression for the flux of tracer into the cylinder, and, in the steady state, it is equal to the flux out of the cylinder through its walls. From Eq. B6 we therefore have:

$$P_d^{\text{cylinder}} = -D\pi R^2 \alpha \left(\frac{e^{-L\alpha} - e^{L\alpha}}{e^{-L\alpha} + e^{L\alpha}}\right). \tag{B7}$$

(Note that P_d^{cylinder} has the dimensions of cubed centimeters per second.)

In a fully stimulated bladder, there are $\sim 10^7$ fusions of tubular vesicles per square centimeter (Levine et al., 1981). Since P_f for the luminal membrane of such a bladder is $\sim 5 \times 10^{-2}$ cm/s (Levine et al., 1984), we have for the hydraulic water permeability coefficient per tubule (cylinder):

$$P_f^{\text{cylinder}} = 5 \times 10^{-9} \text{ cm}^3/\text{s}. \tag{B8}$$

It is easily shown that for this value of P_f the osmotic flow through the walls of a cylinder 2 μm long and 0.1 μm in diameter is uniform throughout its length.[8] It directly follows from this that

$$P_f^{cylinder} = 2\pi RLP_f, \tag{B9}$$

where $P_f^{cylinder}$ is the hydraulic permeability coefficient (in centimeters per second) of the cylinder walls. Substituting Eq. B8 into B9 along with the dimensions of the cylinder (L = 2 μm; R = 0.05 μm), we find that

$$P_f = 0.8 \text{ cm/s}. \tag{B10}$$

This is not an unreasonable permeability for the walls. If their permeability resulted from the short channels of 2 Å radius, discussed in the text, which have a value for p_f of 4.1×10^{-13} cm^3/s (see Eq. 4*a*), then the number of such channels in the wall would be 2×10^{12}/cm^2 or 1 channel/5,000 Å^2.

For the above channels, $p_f = p_d(w)$ and therefore $P_f = P_d$ in the walls of the cylinder. From Eq. B10 this means that P_d = 0.8 cm/s. Substituting this value into Eq. B3 we have:

$$\alpha = 1.1 \times 10^5 \text{ cm}^{-1}$$

and thus for our cylinder of length 2 μm and diameter 0.1 μm, we obtain from Eq. B7:

$$P_d^{cylinder} = 2 \times 10^{-10} \text{ cm}^3/\text{s}. \tag{B11}$$

Combining Eqs. B8 and B11 we finally obtain

$$\frac{P_f^{cylinder}}{P_d^{cylinder}} = 25. \tag{B12}$$

Thus, even if $p_f = p_d(w)$ for the channels in the walls of the cylinder (tubular vesicle), the value of $P_f/P_d(w)$ for the luminal membrane can be large.

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[8] This follows from the fact that the hydraulic permeability coefficient for flow down the cylinder is, from Poiseuille's Law, $\sim 2 \times 10^{-7}$ cm^3/s. Since this is 40 times larger than the actual permeability coefficient (5×10^{-9} cm^3/s), there is only a very small fall in pressure from the top to the bottom of the cylinder, and hence the driving force for water across the walls of the cylinder is essentially constant throughout its length, as long as the mucosal bath (and therefore the cylinder) contains only distilled water. For the low mucosal solute concentrations used in our experiments, solute polarization within the cylinder is so small that the above conclusion is still valid.

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