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The Role of Water Diffusion in the Action of Vasopressin

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Summary. Vasopressin produces a large increase in the osmotic flow of water across the toad bladder, with little apparent change in the diffusion rate of tritiated water. This discrepancy between osmotic and diffusional net flow is the basis of the pore theory of vasopressin action. The present studies show that there is in fact a large (at least 10-fold) increase in water diffusion subsequent to addition of vasopressin, which is masked by unstirred layers and by the resistance offered to diffusion by the thick layer of connective tissue and muscle supporting the bladder epithelial cells. An even higher diffusion rate would be anticipated with the complete elimination of unstirred layers, and of barriers to diffusion remaining within the epithelial layer itself. An alternative to the pore hypothesis is considered, in which vasopressin acts solely by increasing the diffusion rate of water across the luminal membrane of the epithelial cell.

It is generally believed that the osmotic flow of water across living cells is the result of Poiseuille flow through aqueous channels (pores) in the cell membrane [14, 19]. This view is based on the observation that the rate of diffusion of labelled water across cells does not appear to be high enough to account for the large water flows observed. In the isolated urinary bladder of the toad, for example, total osmotic flow exceeds the net flow predicted from the rate of diffusion of tritiated water (THO) by a ratio of 6:1. When the bladder is treated with vasopressin, osmotic flow increases 40-fold, with only a small apparent increase in diffusion, and the ratio of osmotic to diffusional net flow becomes greater than 100:1 [11].

It is possible to arrive at an estimate of the mean radius of the pores required to produce such a discrepancy between osmotic and diffusional net flow by employing the following expressions. For osmotic flow,

$$L_p/\overline{V}_w = \frac{n\pi r^4 \Delta P}{8\eta \Delta x}; \tag{1}$$

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for net diffusional flow,

$$\omega_T = \frac{n \pi r^2 D \Delta P}{R T \Delta x}, \tag{2}$$

where L_p is the hydraulic water flow in the presence of a hydrostatic or osmotic driving force, $\overline{V}_w$ the molar volume of water, n the number of pores, r the mean pore radius, ΔP the gradient of pressure, η the bulk viscosity coefficient of water, Δx the membrane thickness, ω_T the net diffusional flow, D the self-diffusion coefficient of water in water, R the gas constant and T the absolute temperature.

When the terms are combined, common terms cancel out, yielding the expression

$$L_p / \overline{V}_w \omega_T = \frac{r^2 R T}{8 \eta \overline{V}_w D}. \tag{3}$$

Therefore, if $L_p/\overline{V}_w$ and ω_T are determined experimentally, and expressed as a ratio, the mean pore radius can be estimated. The high apparent ratios in the case of the toad bladder yield values for pore radius of 8 A in the absence of vasopressin and approximately 40 A after exposure to the hormone.

This report presents evidence that the rate of diffusion of water across the vasopressin-treated toad bladder (and therefore ω_T) has been greatly underestimated, and that the process of diffusion may play an important role in the action of the hormone. Unstirred layers of water in apposition to the bladder, and the thick layer of muscle and connective tissue supporting the bladder epithelial cells have been found to contribute significantly to the resistance of the bladder to diffusion; when the contribution of these layers is taken into account, the true rate of diffusion of water across the epithelial cells is found to rise strikingly after vasopressin is added.

Materials and Methods

Bladder halves were removed from doubly pithed female Dominican Republic toads (*Bufo marinus*) supplied by National Reagents Inc. (Bridgeport, Conn.). Bladder halves were mounted in lucite chambers with 50 ml phosphate-buffered Ringer's solution bathing each side. The Ringer's solution contained: 120 mM Na^+, 4.0 mM K^+, 0.5 mM Ca^{++}, 116 mM Cl^-, 1.0 mM $H_2PO_4^-$, 4.0 mM HPO_4^{--}; pH 7.4; 230 mosm/kg H_2O. A nylon hair net was stretched across each side of the bladder to hold it rigidly in position during stirring. Stirring was provided by four-bladed paddles cut from Teflon homogenizers; the paddles were attached to Cenco variable-speed stirrers (Central Scientific Co. Mountainside, N.J.), and entered the chambers through holes in the top (Fig. 1). The temperature of the bathing solutions was not raised by the stirring, and therefore was not a factor in the diffusion and flow rates obtained.

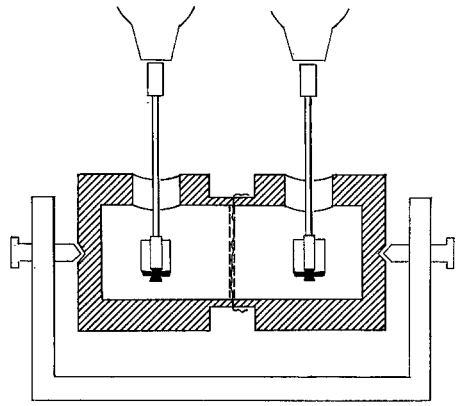

Fig. 1. Chambers used to measure THO diffusion and osmotic flow. A bladder separates the two chamber halves, and is supported on both sides by a fine nylon hair net (dashed lines). Stirring paddles are shown

The rate of diffusion of THO (New England Nuclear, Boston, Mass.) was determined as described previously [11]. Paired bladder halves were studied simultaneously in two sets of chambers set at different stirring speeds. Full-strength Ringer's solution bathed both surfaces of the bladder to eliminate solvent drag effects on THO diffusion; net water flow under these conditions is small (0.004 ± 0.001 ml $\cdot$ cm^{-2} $\cdot$ hr^{-1}). Zero time samples were taken 5 min after the addition of 80 mU/ml of vasopressin (Pitressin; Parke, Davis & Co., Detroit, Mich.) to the serosal side; a second set of samples was taken 30 min after the first. Samples were counted in a Packard scintillation counter (Packard Instrument Co., Downer's Grove, Ill.). Results of the diffusion experiments are expressed as permeability coefficients (K_{trans} THO) [11]. The net water flow predicted from the process of diffusion in mol $\cdot$ dyne^{-1} $\cdot$ sec^{-1} is expressed as ω_T [10]. The total osmotic flow of water after addition of vasopressin was determined as follows [11]: both chamber halves were weighed empty, a bladder half was placed between them, and both chamber halves were then filled with appropriate Ringer's solution, using calibrated pipettes. At the end of the experiment, the chamber contents was removed with preweighed syringes, the chambers were separated and the bladder removed. The Ringer's remaining in the chambers was determined by reweighing the chamber halves. The small amount of Ringer's adhering to the stirrers was determined by blotting them with preweighed tissues and weighing the tissues. Net water movement was calculated from the difference in weight of the solutions at the end of the experiment. In the osmotic flow experiments, half-strength Ringer's solution (115 mosm/kg) bathed the luminal surface, and full-strength Ringer's the serosal surface of the bladder. The time period for these experiments was again 30 min. Total osmotic flow, in mol $\cdot$ dyne^{-1} $\cdot$ sec^{-1}, is expressed as $L_p/\bar{V}_w$, where $\bar{V}_w$ is the molar volume of water [10].

The contribution of the supporting layer of the bladder to the overall rate of diffusion of water was determined by measuring K_{trans} THO across the intact bladder for 30 min, then removing the Ringer's solution and substituting calcium-free Ringer's solution in both chamber halves for a 1-min period. This was repeated twice, and had the effect of loosening the bladder epithelial cells [12]. The chamber halves were then separated, and, with the bladder still stretched across one chamber half, an edge of bladder was lifted and a glass slide gently inserted below the serosal surface. The epithelial cells were thoroughly scraped from the luminal surface of the bladder with a cover slip, leaving

the supporting layer stretched across the chamber. The chambers were placed together and refilled with 50 ml of calcium-containing Ringer's solution. K_{trans} THO across the supporting layer was then determined for 30 min. At the conclusion of the experiment, the supporting layer was removed, placed in 3 % glutaraldehyde, sectioned in paraffin, and examined microscopically to determine the completeness of epithelial-cell removal.

Standard statistical methods, including those for pair analysis, were used [23].

Results

Effect of Stirring on Water Permeability

The effect of the rate of stirring on the permeability of the bladder to THO is shown in Fig. 2. In the vasopressin-treated bladders, K_{trans} THO at first rose sharply as the stirring rate was increased from 135 to 345 rpm; the rise at higher rates was less pronounced, and appeared to be approaching a limiting value at 800 rpm. The values for each stirring rate were highly reproducible, as indicated by small standard errors.

Stirring rate also influenced the permeability of the bladder to THO in the absence of vasopressin, although the effect was not as pronounced (Fig. 2). In four paired experiments, K_{trans} THO increased 35 % as stirring increased from 135 to 800 rpm; the difference was significant ($p < 0.02$).

Included in Fig. 2 are the earlier data of Hays and Leaf [11] for the permeability of THO in the presence and absence of vasopressin. These experiments were carried out in unstirred chambers, in which mixing of the bulk solution was provided by a column of air bubbles. An increase of only 60 % in the permeability coefficient subsequent to addition of vasopressin was noted in these studies, in contrast to the fivefold increase obtained with the present stirring technique.

To rule out injury to the bladders as a factor in the high permeabilities obtained with stirring, the reversibility of the vasopressin effect was studied at a high stirring speed. Two bladders were subjected to 1,200-rpm stirring for 30 min in the presence of vasopressin; after this, the Ringer's solution in both chambers was removed and replaced with fresh solution which did not contain vasopressin. This washing procedure was rapidly carried out three times, and the chambers were then left filled with vasopressin-free Ringer's solution for 40 min. After the chambers were again emptied and refilled, the permeability of the bladders to THO in the absence of vasopressin was determined for a 30-min period, again at 1,200 rpm. The results are shown in Table 1; in both bladders, the permeability returned to baseline values when vasopressin was withdrawn. It could also be shown that, in any given bladder, decreasing the stirring speed from 800 to 130 rpm in the

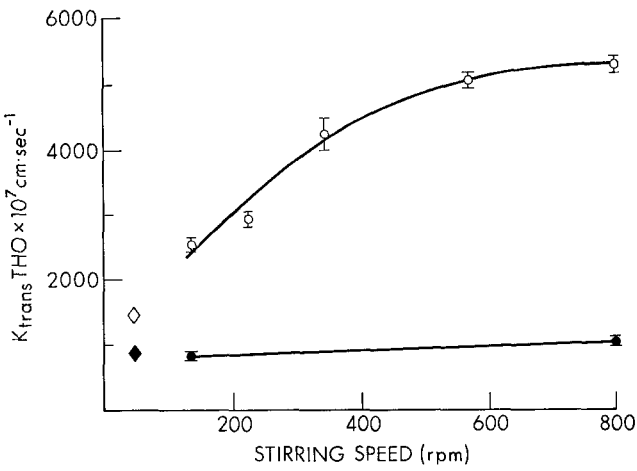

Fig. 2. Effect of stirring rate on K_{trans} THO. Open symbols are vasopressin-treated bladders, closed symbols are control bladders. Diamond symbols are the earlier values of Hays and Leaf [10]. Vertical bars are ± 1 SE

Table 1. *Reversibility of the effect of vasopressin on K_{trans} THO at high stirring speeds*

Experiment	K_{trans} THO[a] ($cm \cdot sec^{-1} \times 10^7$)	
	Vasopressin	Control
1	7,793	1,194
2	7,005	1,192

[a] In both bladders, K_{trans} was first determined in the presence of vasopressin at high stirring speed (1,200 rpm). Vasopressin was then removed, and K_{trans} was redetermined at 1,200 rpm.

presence of vasopressin decreased K_{trans}THO to the same value obtained in the paired experiments shown in Fig. 2 (5,453 and 2,708 $\times 10^{-7}$ cm $\cdot$ sec^{-1}, respectively).

Further evidence that the bladder remained intact during rapid stirring was obtained in experiments with ^{14}C thiourea, a compound which penetrates the bladder slowly, and whose permeability coefficient is unchanged after addition of vasopressin [16]. In six experiments in the presence of vasopressin, the mean K_{trans} for thiourea at 800 rpm was 10.5 ± 4.5 (SE) $\times 10^{-7}$ cm $\cdot$ sec^{-1}, comparable to the value of 14×10^{-7} cm $\cdot$ sec^{-1} obtained in earlier studies [16]. Thiourea movement across paired bladder halves run at lower speeds (225 rpm) also gave a K_{trans} of $10.7 \pm 5.4 \times 10^{-7}$ cm $\cdot$ sec^{-1}, evidence that no significant change in bladder area occurred at high stirring rates.

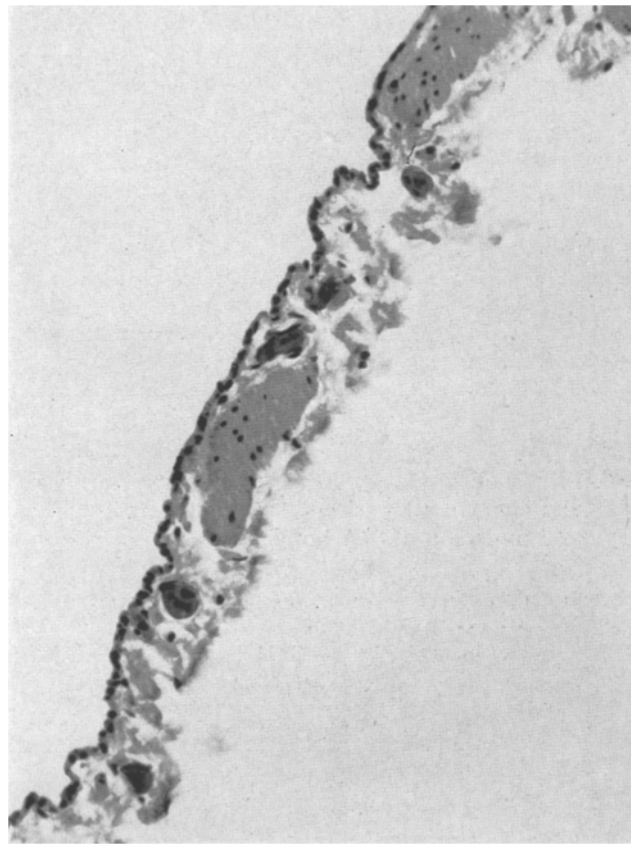

Fig. 3a

Fig. 3a and b. Normal bladder (a) showing intact row of epithelial cells along left border of tissue. After scraping (b), the supporting layer is shown, with the epithelial cells removed. H & E stain. × 240

Effect of Stirring on Osmotic Flow

In contrast to the pronounced effect of stirring on the diffusion rate of water, there was no significant increase in the rate of osmotic flow subsequent to addition of vasopressin when the stirring rate was increased from 225 to

Table 2. *The effect of stirring on the osmotic flow of water in 5 paired experiments*

Speed (rpm)	Osmotic flow[a] ($ml \cdot cm^{-2} \cdot hr^{-1}$)
225	0.158 ± 0.016 (SE)
800	0.176 ± 0.025 (SE)

[a] $\Delta = 0.018 \pm 0.014$. $p < 0.3$.

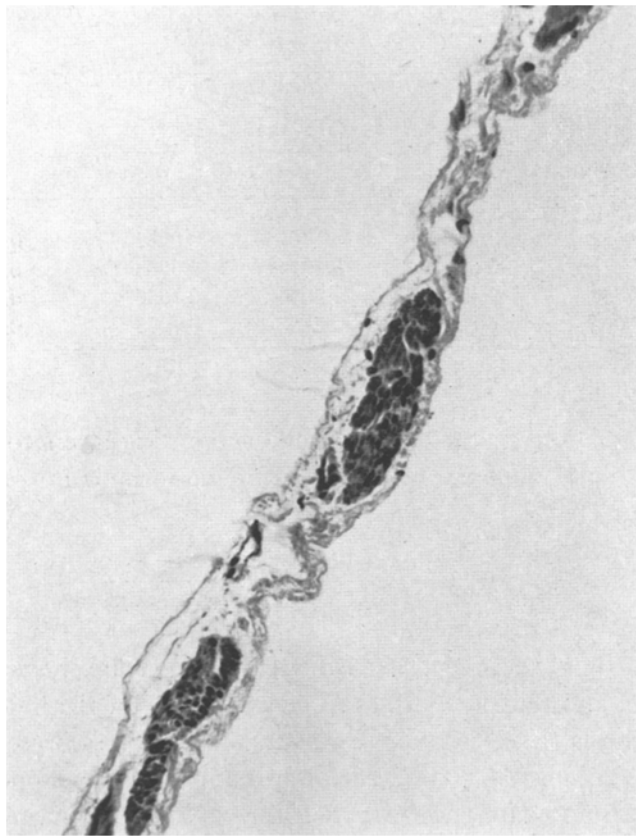

Fig. 3b

800 rpm (Table 2). The maximum water flows obtained in these experiments were comparable to, although somewhat higher than, those obtained in earlier studies with the bubble chamber [11] (0.176 and 0.143 ml · cm^{-2} · hr^{-1}, respectively, at an osmotic gradient of 115 mosm/kg).

Contribution of the Supporting Layer

A supporting layer of smooth muscle, collagen and blood vessels, 20 to 100 μ in thickness, underlies the epithelial cell layer of the bladder. The contribution of this layer to the overall resistance of the bladder to THO diffusion was determined by comparing the K_{trans}THO across the intact bladder to the K_{trans}THO across the supporting layer with the epithelial cells removed (*see* Methods). Representative sections of intact and scraped bladders are shown in Fig. 3.

Table 3. *Contribution of the supporting layer to the total resistance of the bladder to THO diffusion*

Experiment	K_{trans} THO ($cm \cdot sec^{-1} \times 10^7$)		$1/K_{trans}$ THO ($sec \cdot cm^{-1} \times 10^{-4}$)		$\frac{\Omega \text{ Support}}{\Omega \text{ Intact}}$
	Intact	Support	Intact	Support	
1	5,431	10,179	0.1841	0.0982	0.53
2	5,220	10,603	0.1916	0.0943	0.49
3	4,888	9,525	0.2046	0.1050	0.51
4	4,635	9,149	0.2157	0.1093	0.51

The contributions of the epithelial and supporting layers to the overall resistance of the bladder can be expressed by the formula for resistances in series [13, 15]:

$$\frac{1}{K_{trans}\,\text{total}} = \frac{1}{K_{trans}\,\text{epith.}} + \frac{1}{K_{trans}\,\text{supp.}}. \tag{4}$$

The results of four experiments carried out at 800 rpm are shown in Table 3. In the second and third columns, the permeabilities of the intact bladders and supporting layers are listed; these values are expressed reciprocally as resistances in columns 4 and 5. The contributions of the supporting layer to overall resistance are shown in the last column; the mean value was 51 ± 0.8 (SE)%. From these data, one may conclude that the permeability of the epithelial layer alone to water is twice that measured across the epithelial plus supporting layer.

Although the supporting layer offered a significant resistance to diffusion, it presented no appreciable resistance to hydraulic flow. This was shown by placing a supporting layer from which the epithelial cells had been scraped on a disc of a 14-μ-radius, nylon Millipore filter which was supported by a sintered-glass disc (Fig. 4). The assembly was placed on top of a side-arm flask, and a calibrated column with a ground-glass base was clamped on top of the assembly and filled with Ringer's solution. Suction was applied to the side-arm flask with a vacuum pump attached to a mercury gauge, and the flow of Ringer's down through the supporting layer was determined at 1-min intervals for 10 min. The flow remained steady at a rate of 18 $ml \cdot cm^{-2} \cdot hr^{-1} \cdot atm^{-1}$, a value over 400 times greater than that observed in vasopressin-treated intact bladders. When an intact bladder treated with vasopressin was substituted for the supporting layer, water flow was so small as to be undetectable with the apparatus. Hydraulic (or osmotic) flow

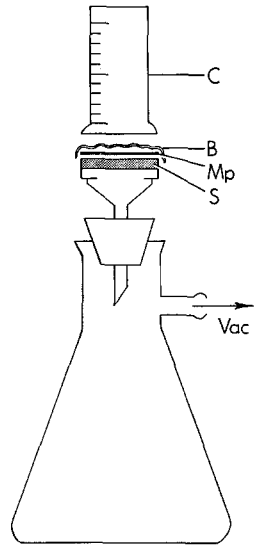

Fig. 4. Apparatus used to determine hydraulic flow across supporting layer. *C*, calibrated column; *B*, bladder supporting layer; *Mp*, Millipore filter; *S*, sintered glass funnel; *Vac*, to vacuum pump

across the supporting layer, therefore, proceeded at such a fast rate as to be of negligible importance when compared to flow across the epithelial layer, in determining overall flow rate.

Discussion

The question considered in these studies is whether the rates of water diffusion and flow, as measured across the intact toad bladder, can be used to estimate pore dimensions in the epithelial cell membrane.

Experience with simple, homogeneous artificial membranes has shown that the ratio $L_p/\overline{V}_w\,\omega_T$ does in fact permit the calculation of mean pore radii which are in good agreement with radii obtained from data on the sieving of small molecules of known radius [21]. However, in more complex membranes, where permeability barriers exist in series, the ratio $L_p/\overline{V}_w\,\omega_T$ may have no relationship to the mean pore radius of any single barrier, but reflects the entire range of radii throughout the membrane. An example is the cellulose acetate reverse-osmosis membrane, which consists of a thin dense "skin", rate-limiting for osmotic flow, and a thick porous supporting layer, rate-limiting for the diffusion of isotopically labelled water [10]. When the retarding effect of the thick supporting layer to K_{trans}THO was taken into account, the true K_{trans}THO across the skin was seen to be 10

times greater than that measured across the whole membrane, and the estimated mean pore radius of the skin was greatly reduced.

The same considerations apply to epithelia such as the toad bladder. Several lines of evidence support the view that the 100-A thick luminal membrane of the epithelial cell is rate-limiting for osmotic flow, and is the ultimate site of action of vasopressin [3, 18, 20]. Most, if not all, of the barriers in series with the luminal membrane, including the epithelial cell cytoplasm, the wide intercellular channels that form in the presence of vasopressin [1, 5], the thick supporting layer, and the unstirred layers, are structures that would be expected to impede diffusion but to offer virtually no resistance to flow. It is uncertain if the basolateral membrane of the epithelial cell is a significant barrier to flow. There is evidence that the basolateral membrane is highly permeable [15][1]; this may reflect a high "porosity", a high intrinsic diffusional permeability, or simply a large surface area.

The present studies demonstrate that unstirred layers in the vasopressin-treated toad bladder are in fact a significant barrier to diffusion. At 800 rpm, K_{trans}THO after addition of vasopressin increased at least fivefold over control values in the hormone-free preparation; this increment would be still higher in the complete absence of unstirred layers, a condition that could not be achieved in these experiments. A relationship of stirring rate to K_{trans}THO comparable to that in the toad bladder has been demonstrated in a variety of synthetic membranes [7] and in thin lipid membranes [6, 9]. Cass and Finkelstein [2] have been able to show that with high stirring rates, K_{trans}THO across thin phospholipid membranes was high enough to account for the entire osmotic flow. Studies of biological membranes have yielded varying results. Dainty and House [4] found that K_{trans}THO across frog skin in the absence of vasopressin rose significantly with increasing stirring rates, but showed no further increment after vasopressin was added. Stirring had little effect on osmotic flow. Gutknecht [8], in studies with *Valonia ventricosa*, found that correction for the unstirred-layer effect gave values for $L_p/\overline{V}_w$ and ω_T which were virtually identical, suggesting that diffusion is the sole mechanism for osmotic water movement in this species. Sha'afi, Rich, Sidel, Bossert and Solomon [22] found little interference by unstirred layers in their studies of the water permeability of erythrocytes; stirring in their system, however, was probably more efficient than in studies with epithelial tissues.

The thick supporting layer, previously assumed to have a negligible effect on K_{trans}THO [11], must also be regarded as a significant diffusion

1 See also DiBona, Civan and Leaf. 1969. *J. Membrane Biol.* **1**:79.

Table 4. *Osmotic and diffusional net flow across the epithelial cell layer (800-rpm stirring speed)*

$L_p/\overline{V}_w \times 10^{14}$ (mol · dyne^{-1} · sec^{-1})	ω_T	$L_p/\overline{V}_w \omega_T$
97.8	4.3	22.7

barrier after addition of vasopressin, providing half the total resistance to water diffusion, but virtually no resistance to flow. In the absence of vasopressin, neither the unstirred layers nor the supporting layer contributes significantly to the diffusional resistance to water, because these barriers are relatively much more permeable to water than is the luminal membrane. In the presence of hormone, the permeability of the luminal membrane rises to the point that the unstirred and supporting layers constitute significant barriers to diffusion.

At 800 rpm, K_{trans}THO across the intact bladder reaches a value of $5{,}300 \times 10^{-7}$ cm · sec^{-1} (Fig. 2). The supporting layer, at this speed, contributes 51 % of the total resistance to diffusion (Table 3); its contribution at infinite stirring speed cannot be estimated from the present experiments, but it would still be substantial. K_{trans}THO across the epithelial layer alone is $1/0.49 \times 5{,}300$, or $10{,}800 \times 10^{-7}$ cm · sec^{-1} at 800 rpm. Vasopressin, then, produces at least a 10-fold increase in water diffusion. If one expresses the value for osmotic and net diffusional flow in comparable terms (mol · dyne^{-1} · sec^{-1}), one obtains the values for diffusion and flow shown in Table 4. The ratio of $L_p/\overline{V}_w \omega_T$ across the epithelial layer is 23:1, compared to the earlier value of 120:1 obtained with the bubble chamber at a comparable osmotic gradient [11].

Although the present ratio of $L_p/\overline{V}_w \omega_T$ is far smaller than originally reported, it is still high, and the mean pore radius calculated from the new ratio is 18 A. If pores this size were opened at the luminal cell surface, it would not be possible to explain the observation that solutes with radii of 3 to 4 A move no faster across the bladder after addition of vasopressin [16]. It would therefore be necessary to retain the "dual-barrier" hypothesis of hormone action, in which a dense diffusion barrier is in series with the vasopressin-sensitive porous barrier; the dense barrier would maintain a low permeability of the bladder to small solutes [11, 17]. To reduce the estimated pore radius to a size compatible with the specificity of the bladder toward small solutes, $L_p/\overline{V}_w \omega_T$ would have to be considerably smaller than 23:1.

K_{trans}THO, therefore, would have to be considerably higher than the present value of $10{,}800 \times 10^{-7}$ cm · sec^{-1}.

It must be emphasized, however, that this value for K_{trans}THO is a minimum one, not only because unstirred layers were not completely eliminated, but because this permeability coefficient applies to the entire thickness of the epithelial cell layer. K_{trans}THO across the luminal cell membrane alone would be expected to be significantly higher. The cell cytoplasm, as well as the dilated intercellular channels which form after addition of vasopressin even without an osmotic gradient [5], are two additional barriers that would be expected to retard diffusion but not flow.[2] The extent to which these barriers would retard diffusion depends on their effective thickness, the area of cytoplasm open to diffusion and the cytoplasmic viscosity; at present, no accurate estimate of these factors can be made. Finally, the luminal cell membrane itself may be complex in structure, with a thin superficial component limiting osmotic flow, and a thicker porous component limiting diffusion [10].

Clearly, experiments are required which would permit the determination of osmotic and diffusional net flows across the luminal cell membrane itself. Under these conditions, the extent to which diffusion contributes to the action of vasopressin could be assessed. A sufficiently high value for diffusion would make large pores unnecessary, and would suggest an alternative mode of action of the hormone: that of opening a great number of aqueous channels of dimensions close to that of the water molecule itself. A second possibility is that vasopressin alters the permeability of the cytoplasm, transforming it from a relatively impermeable and rate-limiting barrier to a more fluid state, where it and the cell membrane contributed to the resistance to water diffusion.

Whether or not these alternative views of hormone action can be supported experimentally, the present studies demonstrate the hazards of estimating cell membrane structure from permeability studies across entire tissues, and indicate that the effect of vasopressin on the rate of diffusion of water is considerably more important than has heretofore been believed.

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2 Although L_p is not affected by unstirred layers, it is possible that the so-called "sweeping away" effect (the dilution of the serosal solution by water flowing across the membrane) may reduce the actual osmotic gradient across the membrane, and lead to an underestimation of L_p. This has been shown to be the case in *Chara australis* (Barry & Hope, 1969. *Biophys. J.* **9**:729) and in gallbladder (Wedner & Diamond, 1969. *J. Membrane Biol.* **1**:92). The magnitude of this effect has not been established in the toad bladder.

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