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Membrane Pathways for Water and Solutes in the Toad Bladder: II. Reflection Coefficients of the Water and Solute Channels

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Summary. Urea and water transport across the toad bladder can be separately activated by low concentrations of vasopressin or 8 Br-cAMP. Employing this method of selective activation, we have determined the reflection coefficient (σ) of urea and other small molecules under circumstances in which the bladder was transporting urea or water. An osmotic method for the determination of σ was used, in which the ability of a given solute to retard water efflux from the bladder was compared to that of raffinose ($\sigma = 1.0$) or water ($\sigma = 0$). When urea transport was activated (low concentration of vasopressin), σ for urea and other solutes was low, (σ_{urea}, 0.08–0.39; $\sigma_{acetamide}$, 0.55; $\sigma_{ethylene\ glycol}$, 0.60). When water transport was activated (0.1 mM 8 Br-cAMP) σ_{urea} approached 1.0. σ_{urea} also approached 1.0 at high vasopressin concentrations. In a separate series of studies, σ_{urea} was determined in the presence of 2×10^{-5} M $KMnO_4$ in the luminal bathing medium. Under these conditions, when urea transport is selectively blocked, σ_{urea} rose from a value of 0.12 to 0.89. Thus, permanganate appears to "close" the urea transport channel. These findings indicate that the luminal membrane channels for water and solutes differ significantly in their dimensions. The solute channels, limited in number, have relatively large radii. They carry a small fraction (approximately 10%) of total water flow. The water transport channels, on the other hand, have small radii, approximately the size of a water molecule, and exclude solutes as small as urea.

In the preceding paper (Carvounis *et al.*, 1979) we have shown that urea or water transport across the toad bladder could be selectively activated by low concentrations of vasopressin or 8-Bromo cAMP, respectively. In the present study, we determined the reflection coefficients (σ) of urea and other solutes when urea or water transport were separately activated. With activation of urea transport, σ for urea and other small solutes was low; with the activation of water transport, σ_{urea} approached 1.0. On the basis of these studies, we propose that water and solute movement across the luminal membrane proceeds through channels whose size and number differ significantly.

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Materials and Methods

Female Dominican toads (National Reagents, Bridgeport, Conn.) were doubly pithed and glass bungs were tied into both hemibladders in situ. The bladders were excised, and washed inside and outside three times with amphibian phosphate Ringer's (110 mM Na^+, 0.5 mM Ca^{++}, 4 mM K^+, 106 mM Cl^-, 5 mM phosphate 230 mosmol/kg, pH 7.40). Methods and materials used for determination of osmotic water flow and permeabilities of various radioisotopic solutes were identical to those described in the preceding paper (Carvounis *et al.*, 1979).

Reflection Coefficient

Figure 1 outlines a method of measuring the reflection coefficient. Consider a situation in which three identical hemibladders are bathed in 220 mosmol/kg buffered NaCl. The first hemibladder is filled with water, the second with 100 mosmol/kg raffinose, a large impermeant solute with a σ of 1.0, and the third with 100 mosmol/kg of a solute (s) whose reflection coefficient (σ) is to be measured. Of the three tissues, bladder 1 would lose water most rapidly, while bladder 2 would have approximately one-half the water flow of bladder 1, since the impermeant raffinose decreased the osmotic gradient by approximately one-half. The water flow in bladder 3 would depend critically on σ_s. If σ_s were

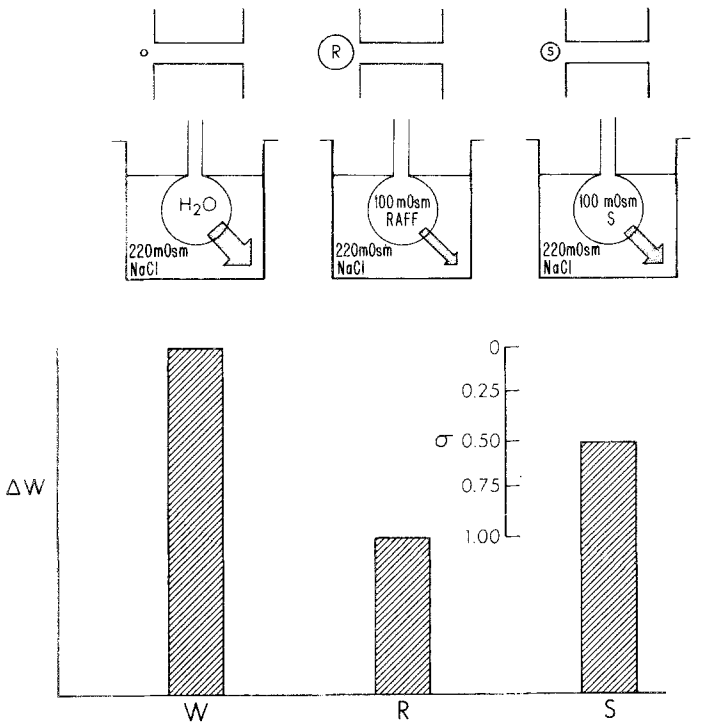

Fig. 1. Schematic representation of the osmotic method of determining reflection coefficients. Water flow is depicted across bladders containing water alone (W); raffinose (R, an impermeant molecule larger than the available channels), and a relatively small solute (S), with a reflection coefficient falling between those of water and raffinose

1.0, it would retard net water efflux to the same extent as an equiosmolar concentration of raffinose. If σ_s were equal to zero, water flow would be the same as in the bladders containing only water. As shown on the right-hand scale in the figure, intermediate values for σ_s would elicit water flows at a rate linearly interpolated between those for $\sigma_s=0$ and $\sigma_s=1$. Thus:

$$\sigma_s=\frac{\Delta W_w-\Delta W_s}{\Delta W_w-\Delta W_r} \tag{1}$$

where ΔW_w, ΔW_s, and ΔW_r are water flows in the tissues containing water, test solute, and raffinose, respectively.

Two considerations are involved in the measurement of σ_s in the toad bladder. First, the toad has only 2 hemibladders, so that dividing the tissue into 3 or more segments would preclude using the well-characterized Bentley technique (Bentley, 1958). Second, the water flow response of the bladder to vasopressin varied with time. We dealt with these problems by comparing in paired experiments and in random order the effects of water *vs.* raffinose on transport during one test period, and the effects of water *vs.* test solute during the other test period, calculating reflection coefficient as

$$\sigma_s=\frac{\left(\frac{\Delta W_w-\Delta W_s}{\Delta W_w}\right)}{\left(\frac{\Delta W'_w-\Delta W_r}{\Delta W'_w}\right)} \tag{2}$$

where ΔW_w is the water flow in the bladders containing water during the period in which ΔW_s was measured, and $\Delta W'_w$ the value in the same bladder when ΔW_r was measured. This calculation normalizes for the effects of variation on time and bladder matching on vasopressin-stimulated water flow.

The experiments were carried out by bathing both hemibladders in a serosal solution of full strength Ringer's. One bladder contained 8 ml distilled water (buffered to pH 7.4 with 1 mM Na phosphate) while the paired bladder contained 0.1 M raffinose or 0.1 M test solute. There was a 15 min baseline period in which both sacs were filled with water and osmotic flow was determined gravimetrically. Blotting of the bladder with Whatman # 50 blotting paper was carefully carried out; the small amount of water adhering to the glass bung was also removed. The mucosal solutions were then removed, distilled water was again placed in the first bladder, and 0.1 M raffinose or test solute (whichever was not present in the preceding period) was placed in the second bladder. As indicated above, the order of r or s was randomized. Mechanical stirring was not provided in the experiments to be reported, since we found that in paired experiments at 1 mU/ml vasopressin, identical σ's were obtained in the presence or absence of stirring. We also observed that at high concentrations of vasopressin, stirring produced a decrease in $\sigma_{\text{raffinose}}$ which progressed with time, suggesting a change in the properties of the epithelium.[1]

[1] We did not correct our calculations of water transport rates in the bladders containing urea for the weight change attributable to urea efflux, since urea permeability was not measured in each experiment. Thus, the actual water efflux is in fact slightly less than the bladder's weight loss. The discrepancy, however, is extremely small, on the order of 1%. If we assume a k_{trans} urea of 100×10^{-7} cm/sec in the unstimulated bladders, and k_{trans} urea of 400×10^{-7} cm/sec in the presence of 1 and 86 mU/ml vasopressin, then the bladder weight change would overestimate water loss by only 0.07 and 0.28 mg/min, respectively, when 100 mM urea was present in the bladders' luminal bath. In the tissues containing water or raffinose, water loss and weight change should be essentially identical.

It eventually became clear that the mean value for the denominator of Eq. (2) was 0.50 ± 0.01, ($n=108$) consistent with $\sigma_{\text{raffinose}}=1.0$ and $\sigma_{\text{NaCl}}=0.9$, so that the osmotic effect of the serosal bath was $0.9 \times 230=207$ or approximately the equivalent of 200 mosmol raffinose. Because of the consistency of this value, we chose in some experiments to compare water flows in bladders containing test solute to those containing either water or raffinose in a single period only, calculating σ as:

$$\sigma_s=\frac{\Delta W_w-\Delta W_s}{0.5\,\Delta W_w} \tag{3}$$

or (substituting $2\Delta W_r$ for ΔW_w):

$$\sigma_s=\frac{2\Delta W_r-\Delta W_s}{\Delta W_r}. \tag{4}$$

Experimental results were evaluated by the method of paired analysis (Snedecor & Cochran, 1967).

Results

Effect of Vasopressin on σ_{urea}

The reflection coefficient of urea was determined in 5 paired experiments in which one hemibladder contained buffered distilled water, and

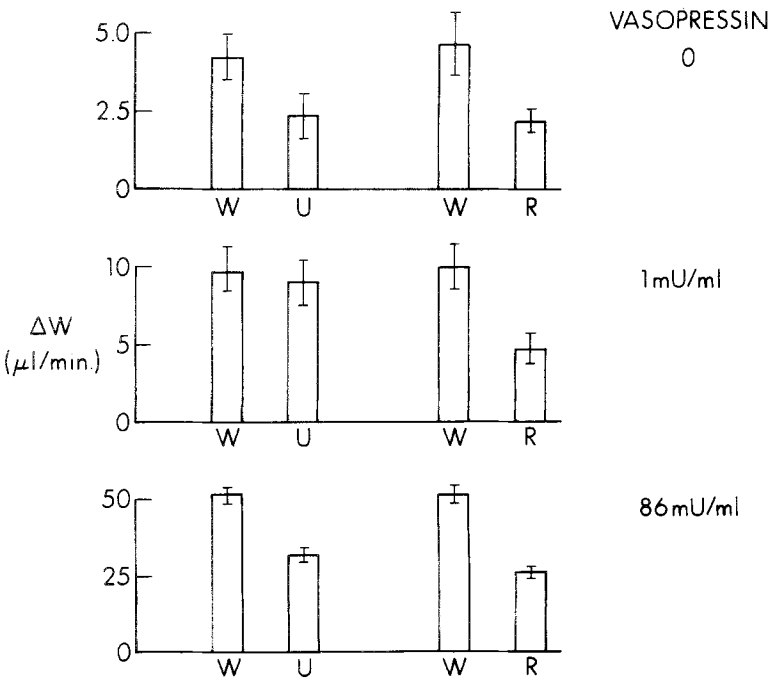

Fig. 2. Water flow across paired bladders, containing water *vs.* urea, or water *vs.* raffinose, in the absence of vasopressin, and with 1 and 86 mU/ml vasopressin. *See* text

the paired bladder contained 0.1 M urea followed by 0.1 M raffinose, or raffinose followed by urea. The bladders were bathed in Ringer's solution containing no vasopressin, 1 mU/ml, or 86 mU/ml vasopressin. The order of exposure to vasopressin (or no vasopressin) was randomized, and extensive washout periods separated periods of vasopressin treatment. The results are shown in Fig. 2. In the absence of vasopressin, urea retarded water flow as effectively as raffinose; σ_{urea} calculated from Eq. (2) was 1.02 ± 0.23 (SEM). Following 1 mU of vasopressin, urea became almost ineffective in retarding water flow; water flow in the sac containing urea was 95% of that in the absence of solute, and σ_{urea} was 0.05 ± 0.10. Raffinose remained an osmotically effective solute. Following 86 mU/ml vasopressin, urea regained much of its osmotic effect with a σ of 0.87 ± 0.07. The results for 1 and 86 mU/ml were confirmed in a larger series in which σ_{urea} following 1 mU/ml was 0.26 ± 0.06 ($n=48$) and following 86 mU/ml was 0.79 ± 0.06 ($n=15$).

Effect of Vasopressin on $\sigma_{acetamide}$ *and* $\sigma_{ethylene\ glycol}$

The reflection coefficients of several small solutes are shown in Fig. 3, in the absence of vasopressin, and following 1 and 86 mU/ml of hormone.

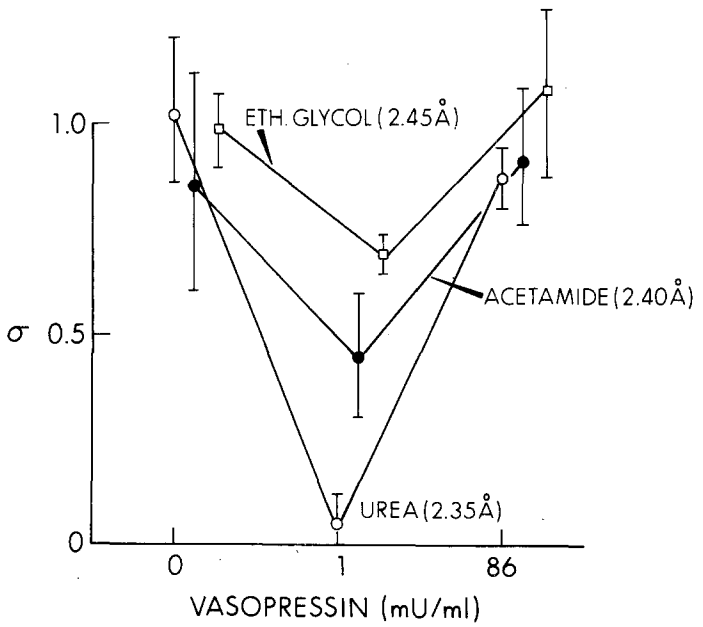

Fig. 3. Reflection coefficients of urea, acetamide ($n=8$), and ethylene glycol ($n=10$) in the absence of vasopressin, and with 1 and 86 mU/ml vasopressin. Molecular radii are shown. Bars are ± 1 SEM

These experiments, like the preceding ones, were done in random order. The data for urea are those obtained in the preceding section (Fig. 2). Acetamide and ethylene glycol showed the same pattern as urea: a significant decrease from baseline values, and a significant rise from 1 to 86 mU/ml vasopressin. The values of σ at 1 mU/ml are a function of the molecular radii of the solutes, as indicated in the figure.

Effect of 0.1 mM 8-Br cAMP on σ_{urea}

As shown in the preceding paper (Carvounis *et al.*, 1979), 0.1 mM 8-Br cAMP activates water flow, but has virtually no effect on urea transport. One would predict that if the water pathway were exclusively activated, σ_{urea} would approach 1.0. This was the case as shown in Fig. 4 and Table 1. When compared to raffinose, urea was almost identical as an osmotic agent; when compared to water, urea showed a substantial osmotic effect. σ_{urea} was 0.86 ± 0.09 (urea *vs.* raffinose, Eq. (4)), and 0.93 ± 0.09 (urea *vs.* water, Eq. (3)). These values were not statistically different from 1.0. To be certain that 0.1 mM 8-Bromo cAMP maintained its selective effect on water flow under the conditions of the osmotic experiments, the permeability of the bladders to ^{14}C urea was simultaneously determined in each of the series described above. There was

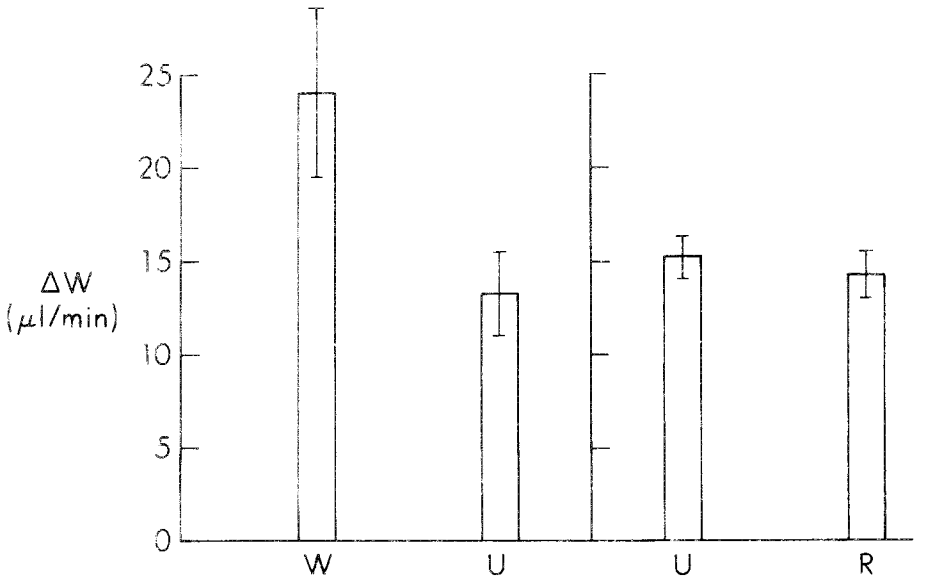

Fig. 4. Water flow across paired bladders, containing water *vs.* urea, or urea *vs.* raffinose, in the presence of 0.1 mM 8-Br cAMP, a selective activator of water flow. Urea is as effective an osmotic agent as raffinose

Table 1. Effect of 8-Br cAMP on σ_{urea}

8-Br cAMP (mM)	Solute (8)[a] None	Urea	σ_{urea}	Solute (6) Raffinose	Urea	σ_{urea}
	ΔW (μl/min)			ΔW (μl/min)		
0	3.8±0.4	4.1±0.6		2.9±0.3	3.2±0.3	
0.1	24.0±4.9	13.4±2.6[b]	0.93±0.09	14.3±1.2	15.5±0.8	0.86±0.09
	k_{trans} urea ($\times 10^7$ cm/sec)			k_{trans} urea ($\times 10^7$ cm/sec)		
0	54 ±22	77 ±37		86 ±25	90 ±23	
0.1	112 ±38	128 ±59		159 ±36	142 ±35[b]	

[a] Number of paired experiments.
[b] Significantly different from paired value.

a small (1.8-fold) increase in the mean k_{trans} urea compared to the baseline values following 8-Br cAMP (Table 1, lower half). This represented a relatively insignificant increase in urea permeability when compared to the 22-fold increase in k_{trans} urea following 1 mU vasopressin (Table 1, Carvounis *et al.*, 1979). Thus, the relative specificity of the activation of water flow by 8-Br cAMP was preserved in these experiments.

Effect of $KMnO_4$ on σ_{urea}

$KMnO_4$ selectively inhibits vasopressin-stimulated urea transport (Franki, Einhorn & Hays, 1975). We determined the effect of $KMnO_4$ on σ_{urea}, using the following protocol: σ_{urea} following 1 mU/ml of vasopressin was first determined in the usual fashion, comparing a hemibladder containing 0.1 M urea with one containing water. The vasopressin effect was then reversed by washing the bladder in hormone-free Ringer's solution and the experiment repeated with 2×10^{-5} M $KMnO_4$ in the serosal bathing medium of both bladders, in the presence of a new dose of 1 mU/ml vasopressin. This experimental design eliminated any effect of $KMnO_4$ on water *per se*, since the agent was present in both baths, $KMnO_4$ produced a consistent and significant increase in σ_{urea}, in addition to reducing the effect of vasopressin on k_{trans} urea. In seven paired experiments, σ_{urea} rose from a value of 0.12 ± 0.25 in the control period to 0.89 ± 0.13 in the presence of $KMnO_4$. The difference was significant by pair analysis ($P < 0.02$).

To be certain that the rise in σ_{urea} was not simply the result of washing out hormone and repeating the first period, a series of 6 experi-

ments was carried out in which the first period was repeated after washout, without adding $KMnO_4$. σ_{urea} was 0.41 in the first period and 0.47 in the second; $\Delta = 0.06 \pm 0.22$, not significant. Thus, a time-dependent rise in σ_{urea} is ruled out.

Discussion

In the preceding paper (Carvounis *et al.*, 1979) the evidence for independent pathways for water and urea transport was reviewed. Not only can the two transport systems be independently blocked or stimulated by appropriate agents, but there is now evidence that vasopressin-induced particle aggregates in the luminal membrane are engaged only in water transport (Kachadorian *et al.*, 1977; Levine *et al.*, 1979). To this evidence, we have added the observation that water or urea transport can be independently stimulated by low concentrations of 8-Br cAMP or vasopressin.

The ability to activate these transport systems independently has enabled us to examine their physical properties, notably the σ_{urea} for each pathway. We have employed an osmotic method for the determination of σ_{urea}. The osmotic method was also employed in an earlier study in the toad bladder by Hays, Harkness and Franki (1970). These studies were limited to an examination of σ_{urea} in the absence of vasopressin and in the presence of a saturating concentration of hormone, with complete activation of both urea and water transport. Mechanical stirring was also provided. Under these conditions baseline σ_{urea} was lower than in the present study, possibly because of less fastidious blotting technique. σ_{urea} following 86 mU/ml vasopressin was 0.70, within the range of values obtained for a saturating concentration in the present study. The sharp fall in σ_{urea} following 1 mU/ml vasopressin, and the high value for σ under conditions in which water flow alone was activated (8 Br-cAMP, $KMnO_4$) were not examined in the earlier study.

The osmotic method employed in the present study is based on the relationship between water flow across the epithelial cell membrane and the "retarding" effect of a given solute on water flow. The model underlying the interpretation of our findings has been shown in Fig. 1. However, in considering the pathways for water and solute movement across the cell membrane, one must evaluate several possibilities. First, water and solutes might both cross the membrane by a process of simple diffusion, in which both species "dissolved" in the membrane phase. Under these

circumstances, vasopressin might act by increasing membrane fluidity (Pietras & Wright, 1975). There is little support for this model; the change in membrane fluidity required for the large increase in water flow would be substantial (Finkelstein, 1976), while the actual change, as determined by fluorescence polarization, is very small (Masters, Yguerabide & Fanestil, 1978). Second, water may traverse small aqueous channels, while urea and other small solutes diffuse through a nonaqueous phase of the membrane. Again, there is some evidence against this model. Vasopressin-stimulated urea transport across the toad bladder has the characteristics of cotransport (Levine & Worthington, 1976), consistent with movement through aqueous channels. In addition, σ_{urea} for a nonaqueous pathway would be 1.0, when appropriate corrections are made for the small difference between weight loss and water (*see* methods).

From these considerations, an aqueous pathway for water and a nonaqueous pathway for urea seems unlikely. The reflection coefficients obtained in these studies, then, represent the effective osmotic presence exerted by urea and other solutes across at least two types of aqueous channels: a relatively large one, activated by low concentrations of vasopressin, in which urea and water interact, and a channel whose radius approximates that of the water molecules, and which excludes urea.

It is important in those osmotic experiments in which 0.1 M concentrations of urea and other solutes were present in the luminal bathing medium to be certain that the integrity of the bladder epithelium was maintained. Na_2SO_4 Ringer's plus 0.2 M urea on the outside of the frog skin will open tight junctions (Ussing, 1963; Ussing & Windhager, 1964). We therefore compared the rate of movement of ^{14}C urea across sacs containing 0.1 M unlabeled urea to ^{14}C urea movement across paired sacs containing buffered distilled water, following 1 mU/ml/vasopressin. Urea permeability was no higher in the sac containing urea than in the sac containing water; indeed, it was significantly lower in 14 paired experiments (132 *vs.* 164×10^{-7} cm/sec, $P < 0.02$) as would be expected with a saturable transport system for amides (Levine, Franki & Hays, 1973).

Turning to the specific findings in this study of reflection coefficients, we obtained values close to 1.0 for σ_{urea}, $\sigma_{acetamide}$, and $\sigma_{ethylene\ glycol}$ in the unstimulated bladder. We recognize, of course, that the osmotic method depends on an accurate determination of water loss from the bladder sacs, and that water flow in the absence of vasopressin is low. This accounts for the relatively large standard error in this set of measurements. However, the consistency of the findings from series

to series suggests that σ_{urea} is indeed high in the unstimulated bladder. We would conclude that, in the absence of vasopressin, the luminal membrane presents few, if any, aqueous channels which are accessible to urea or other solutes and that urea is as effective an osmotic agent as raffinose. The urea that does cross the bladder in the unstimulated state may do so by simple diffusion, although the kinetics of urea transport in the absence of vasopressin have not been thoroughly studied. In addition, it is not clear to what extent solute transport is transcellular or paracellular in the unstimulated bladder.

In the presence of 1 mU/ml vasopressin, σ for all solutes tested fell significantly (Figs. 2 and 3). Again, there was variation in the measured values for σ, with those for urea ranging from 0.08 to 0.39. Even at its highest, however, σ_{urea} was significantly below its baseline value, and below the value obtained with 86 mU/ml vasopressin. The variation in σ for all three solutes was not only the result of experimental error in measuring the low water flows at 1 mU/ml vasopressin, but probably of variation in the extent to which narrow water-transporting channels were activated along with wider solute channels. To the extent that this took place in a particular experimental series, σ would be higher. We have noted some variation in the response of urea and water transport to low concentrations of vasopressin over the course of a year, a phenomenon that may be seasonal in nature.

The question of whether all small hydrophilic nonelectrolytes move through the same channel, or whether amides, for example, traverse a specialized channel, has not been approached in this paper. The finding that σ's for urea, acetamide, and ethylene glycol at 1 mU/ml vasopressin fall in an order predicted from their molecular radii is consistent with a common channel for all 3 solutes. However, an extensive paired series of experiments, employing many solutes, would be required to substantiate this observation. We can conclude that at 1 mU/ml vasopressin, the σ for solutes is determined at least in part by the dimensions of a membrane channel (or channels) involved in solute transport. A small fraction of total water flow takes place through these channels. From Fig. 1 in the preceding paper (Carvounis *et al.*, 1979), we would estimate this fraction of flow to be approximately 10%.[2]

[2] It is of interest in this connection that in one experimental series employing 1 mU/ml vasopressin, $KMnO_4$ reduced water flow by 4 µl/min. This small effect would be masked by the large water flows (40–50 µl/min) following 86 mU/ml vasopressin. If further studies confirm this observation, it would be consistent with the 10% of water flow taking place through the urea channels, and with permanganate blocking transport of both water and urea through these channels.

Both urea and water transport are maximally activated at 86 mU/ml vasopressin. σ_{urea}, as well as that for the other solutes, should rise to a level that reflects the relative proportion of large solute channels and narrow water channels. This is the case, as shown in Fig. 3.

Direct examination of σ for the water channels was made possible by the use of 0.1 mM 8 Br-cAMP, a selective activator of water flow, and of $KMnO_4$, a selective inhibitor of urea transport. In both instances, σ_{urea} rose to levels close to 1.0. The fact that σ_{urea} following 8 Br-cAMP was slightly below 1.0 is explained by the modest activation of urea transport by this synthetic nucleotide (Table 1). $KMnO_4$ also produces a σ_{urea} slightly below 1.0 (0.89), again consistent with the persistence of some urea transport. $KMnO_4$ exerts its inhibitory effect on urea transport at a point beyond the generation of cyclic AMP (Franki *et al.*, 1975) and probably at the luminal membrane itself. It is a potent oxidizing agent, and may alter urea transport channels by irreversibly changing their conformation, or by physically blocking the channels. The experiments with 8 Br-cAMP and $KMnO_4$ support the view that the channels engaged in water transport exclude molecules as small as urea, and have radii in the range of 2.0. Å. Rosenberg and Finkelstein (1978) have shown that gramicidin channels in lipid bilayers have these properties, and that water molecules traverse these channels in a "single-file" fashion.

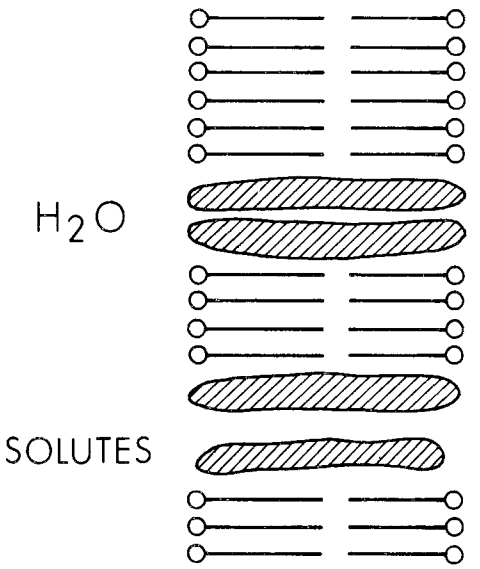

Fig. 5. Vasopressin-generated channels in luminal membrane. Narrow channel (top) transports water only: urea channel (bottom) transports solutes and a small fraction of total water flow

In summary, our findings provide evidence for at least two populations of vasopressin-generated channels in the luminal membrane of the toad bladder epithelial cell (Fig. 5). The first, which transports water exclusively, has a radius small enough to exclude urea. Morphologically, it is identified with the aggregated membrane particles seen in freeze-fracture studies of the luminal membrane of amphibian bladder and mammalian collecting duct (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & DiScala, 1975; Harmanci *et al.*, 1977). The second, which transports urea (and possibly a large number of small hydrophilic nonelectrolytes) has a larger radius, of the order of 3–4 Å. This membrane component, presumably a membrane protein, has not yet been identified morphologically: current evidence (Kachadorian *et al.*, 1977) suggests that it is anatomically distinct from the water-transporting aggregates.

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