

Osmotic Regulation of Toad Bladder Responsiveness to Neurohypophyseal Hormones

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ABSTRACT The effect of dilution of the interstitial fluids on the responsiveness of the toad urinary bladder to antidiuretic hormones has been examined *in vivo* and *in vitro*. Toads were given periodic injections with vasopressin while in water so that their plasma osmolality fell below 190 mosmoles/kg H₂O. The hydraulic conductivity of bladders which had been removed from the animal and fixed with 1% glutaraldehyde was 10-fold less in overhydrated toads than in normally hydrated controls. A similar inhibitory phenomenon was observed in *in vitro* studies, when the tonicity of Ringer's fluid in which the bladders were suspended was lowered from its isotonic value. Mannitol, but not urea, could be effectively substituted for one-half of the NaCl content of Ringer's fluid. In other experiments it has been shown that the responsiveness of the bladder to vasotocin is depressed during bulk water movement across the tissue. This "flux inhibition" was found to depend upon the velocity and the duration of water flow from mucosa to the serosa. It is suggested that the responsiveness of the toad bladder to antidiuretic hormones diminishes as the effective osmotic pressure of the interstitial fluids declines.

INTRODUCTION

In the toad the osmotic composition of body fluids is thought to be regulated largely by the neurohypophyseal hormone, 8-arginine-vasotocin, the octapeptide which increases the hydraulic conductivity of the skin and bladder so that water uptake from the external environment is enhanced, and water loss in the urine is diminished (4). The hydraulic conductivity of the urinary bladder remains very low as long as the plasma osmolality is less than 300 mosmoles/kg H₂O; however, when the plasma becomes concentrated beyond this osmotic threshold, the hydraulic conductivity of the bladder increases rapidly and by as much as 60 times its basal value (10). Although the increase in hydraulic conductivity of the bladder during dehydration probably depends upon an increase in the level of vasotocin in the blood (3), the very low

hydraulic conductivity of the bladder during periods of overhydration may not always be due to low circulating levels of hormone alone.

The concept has been entertained in the present study that bladder responsiveness to antidiuretic hormones is regulated by the tonicity of the plasma. Both in vivo and in vitro experiments have shown that the bladder becomes progressively less responsive to hormone as the osmotic pressure of the interstitial fluids falls below 250 mosmoles/kg H₂O. Decreased responsiveness to antidiuretic hormone has also been observed in mammals when vasopressin is administered over prolonged periods and water intake is not curtailed (6, 17, 18). One possible explanation for the phenomenon of vasopressin "escape" is that overhydration increases tubular resistance to vasopressin (12, 18). Another possibility is that renal receptor sensitivity to vasopressin is not impaired in chronically overhydrated mammals, but that dilution of the cortico-medullary osmotic gradient diminishes the antidiuretic response (6). The toad bladder has been studied here as a possibly valid model for the changes in hydraulic conductivity that take place in the distal mammalian nephron when the interstitial fluids are diluted.

MATERIALS AND METHODS

In Vivo Experiments

The in vivo effects of vasopressin on the permeability to water of the toad's urinary bladder were measured with the glutaraldehyde-fixation technique detailed previously (10). Female toads of the species *Bufo marinus* weighing between 200 and 250 g were purchased during the summer months from the National Reagents Inc., Bridgeport, Conn. Animals were kept at room temperature either in tap water or on moist peat moss for 0-3 days. Toads from both groups were injected every 12 hr intramuscularly in the thigh with 10 U vasopressin (approximately 50 mU/g body weight minus urine). An additional 10 U vasopressin was administered into the dorsal lymph sac 1 hr before pithing the toad. A urine sample was aspirated from the bladder neck for cryoscopy. One of the two half-bladders was then rapidly isolated and tied to the end of a glass cannula, so that the mucosa formed the inside and the serosa the outside surface of the bladder sac. The bladder was filled with 7 ml of 1% glutaraldehyde in 0.05 M cacodylate buffer and suspended in a bath of Ringer's fluid. After the bladder's mucosa had reacted with fixative for 5 min at room temperature, the fixative was removed, and the bladder was rinsed twice with Ringer's fluid. The fixed bladder was filled with 7 ml of Na-free Ringer's fluid (30 mosmoles/kg H₂O) and suspended in a 100 ml bath of full strength Ringer's fluid (230 mosmoles/kg H₂O) so that the initial osmotic pressure difference between the mucosal and the serosal solution was 200 mosmoles/kg H₂O. Net water flux along the osmotic gradient (i.e. in the mucosal to the serosal direction) was estimated from the net weight loss of the bladder assembly during the first 15 min after fixation of the bladder. Net water flux was usually maximal during the first 15 min interval, particularly when the bladder had been fixed in a highly permeable state. Bladders were weighed in

dry air with an accuracy of ± 5 mg. The hydraulic conductivity of the fixed bladder was determined from the net water flux, the mean osmotic pressure difference during the 15 min interval, and the bladder surface area estimated volumetrically. The mean osmotic pressure difference varied between 175 and 197 mosmoles/kg H_2O depending upon the outward rate of water flux and the movement of NaCl into the mucosal fluid. The surface area of the bladders used in this study was 16–18 cm^2 . Blood samples for cryoscopy were aspirated from the heart. The interval between pithing the toad and exposing the mucosa of its urinary bladder to fixative was less than 3 min.

In Vitro Experiments

The hydroosmotic effects of neurohypophyseal hormones on the permeability to water of the toad bladder *in vitro* were studied with Bentley's classical method (2). In order to distinguish between the effects of a hypoosmolar solution on the hormone's action on membrane permeability and the effects of a hypoosmolar solution on osmotic flux of water across the membrane, the glutaraldehyde-fixation technique employed previously in temperature studies (9) was again used here. Accordingly, isolated bladders were exposed to neurohypophyseal hormones in isotonic and in hypotonic media, and in the presence or absence of net water movement; they were then fixed by treating the mucosal surface with 1% glutaraldehyde in 0.05 M cacodylate buffer for 5 min. The hydraulic conductivity of the fixed bladder was measured in a tissue bath as detailed above, except that 4–5 hemibladders were usually placed into one 200 ml serosal fluid bath (see Table II). The hydraulic conductivity of the fixed bladder was taken as an index of the capacity of neurohypophyseal hormones to increase the permeability to water of the toad bladder under the given set of conditions before fixation. When the hormone challenge was standard, a change in hydraulic conductivity, therefore, implied a change in bladder responsiveness to hormone secondary to the change in bathing fluids used during the hormone challenge period. The bathing fluids tested were: (a) hypotonic, low NaCl-Ringer's fluid on both the serosal and the mucosal bladder surface (Fig. 2), (b) isotonic Ringer's fluid in which 55 mM NaCl was replaced by 110 mM mannitol or 110 mM urea on both bladder surfaces (Fig. 3), (c) isotonic, 110 mM NaCl-Ringer on the serosa, but 0, 55, 75, or 110 mM NaCl-Ringer on the mucosa (Fig. 4, Fig. 5, Table II).

The Ringer's fluid employed in this study had the following composition, in millimoles/liter: NaCl, 110; KCl, 3.5; $CaCl_2$, 1; $MgCl_2$, 1; dextrose, 5.5; Trizma (Sigma Chemical Co., St. Louis, Mo.), 10; pH, 8.3; osmolality, 230 ± 4 mosmoles/kg H_2O . Na-free Ringer had all the above constituents except for 110 mM NaCl; the pH was 8.3 and the tonicity 30 ± 1 mosmoles/kg H_2O . Aqueous Pitressin (Parke, Davis & Company, Detroit, Mich., Lot No. KL109) was used as a vasopressin source in the *in vivo* studies. Synthetic oxytocin, 1-deamino-oxytocin, and 8-arginine-vasotocin were generously supplied by Dr. R. Walter. Both oxytocin and vasotocin were assayed against a 1-deamino-oxytocin primary standard with the hydro-osmotic toad bladder assay (8) and found to possess full biological activity. These peptides were added only to the fluids bathing the serosal surface of the bladder and only at concentrations required to elicit a maximal hydroosmotic response.

RESULTS

Diminished Response of Overhydrated Toads to Vasopressin

Toads were overhydrated by placing them in tap water and injecting them with vasopressin. Their plasma osmolalities declined from an initial value of 250 mosmoles/kg H₂O to levels as low as 158 mosmoles/kg H₂O over a period of 3 days. As is shown in Fig. 1, the decline in plasma osmolality was associated with a progressive decline in the hydraulic conductivity of the toad's urinary bladder. Hydraulic conductivities of bladders decreased by 90% as the plasma osmolality fell from 250 to about 200 mosmoles/kg H₂O. A further decline in plasma osmolality from 200 to 158 mosmoles/kg H₂O was not associated with a significant reduction in hydraulic conductivity.

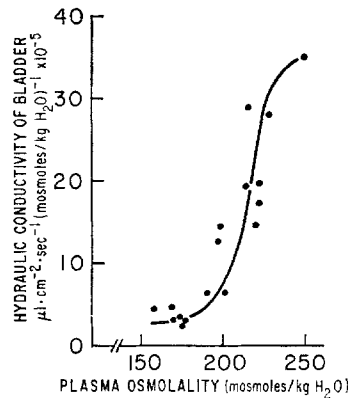


FIGURE 1. Vasopressin resistance in overhydrated toads. Each point on the graph represents the *in vivo* response of the urinary bladder of a single toad to an injection of 10 U Pitressin.

Since it was necessary to inject animals chronically with intramuscular vasopressin in order to overhydrate them, the diminished response of the bladder to hormone could have resulted from the prolonged administration of hormone rather than from the associated plasma dilution. Therefore, another group of toads was also injected every 12 hr with vasopressin for 3 days, but these animals were kept on moist peat moss instead of being kept in water. These toads had plasma osmolalities in the range 250–305 mosmoles/kg H₂O; the hydraulic conductivity of their bladders was about 10 times that observed in overhydrated toads (Table I). Thus, the diminished responsiveness of the bladder to vasopressin is due to factors associated with overhydration and not due to prolonged administration of vasopressin.

In Vitro Response of Bladders to Vasotocin in Hypotonic Media

Experiments were carried out to determine the effects of a hypotonic serosal and mucosal medium on the responsiveness of isolated bladders to vasotocin.

TABLE I
TOAD BLADDER RESISTANCE TO VASOPRESSIN IN VIVO

Toad No.	Environment*	Osmolality		Hydraulic conductivity of fixed bladder†	
		Plasma	Urine		
		<i>mosmoles/kg H₂O</i>		$\mu\text{l} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{mosmoles/kg H}_2\text{O})^{-1} \cdot 10^{-3}$	
1	Tap water	158	—	4.4	
2		169	73	4.6	
3		170	165	3.1	3.9±0.5§
4		174	—	3.4	
5		175	—	2.3	
6		176	52	3.3	
7		190	117	6.5	
<i>P</i> <0.001					
8	Moist peat moss	250	172	35.3	
9		281	270	39.9	
10		288	259	46.1	37.1±2.8§
11		304	296	29.3	
12		305	295	34.8	

* Toads received 10 U aqueous Pitressin intramuscularly every 12 hr over a 3 day period, and an additional 10 U into the dorsal lymph sac 1 hr before fixing the urinary bladder.

† The hydraulic conductivity of fixed bladders was measured under standard conditions, see text.

§ Mean and standard error of the mean.

Bladders were exposed to the same hypotonic Ringer's fluid on both surfaces so that there was no net water movement across the bladder wall. Saturating concentrations of vasotocin (1×10^{-7} M) were added to the serosal medium for 30 min and the bladder was then fixed with glutaraldehyde. As can be seen in Fig. 2, the hydraulic conductivity of the fixed bladder declined progressively as the tonicity of the Ringer's fluid was lowered from 230 to 50 mosmoles/kg H₂O by omitting increasing amounts of NaCl. Thus, the responsiveness of the isolated bladder to vasotocin diminishes with a reduction in the osmolality of the extracellular fluids.

Bladder Response to Vasotocin in Mannitol- and Urea-Ringer

A series of experiments was carried out to see whether bladder responsiveness to vasotocin was preserved when half the usual concentration of NaCl in Ringer's fluid was replaced by equiosmotic concentrations of either mannitol or urea. The results in Fig. 3 show that 110 mM mannitol effectively substitutes for 55 mM NaCl in Ringer's fluid during a 30 min period of exposure to 1×10^{-7} M vasotocin. On the other hand, 110 mM urea was not as effective as either NaCl or mannitol, since the responsiveness of the bladder was diminished by about 50%. It is noteworthy, however, that urea was partially effective in preventing the inhibition seen in hypotonic media because the

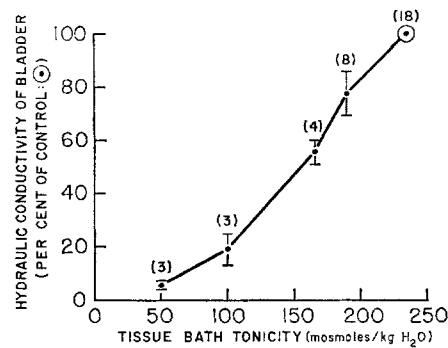


FIGURE 2. Diminished bladder responsiveness to vasotocin (10^{-7} M) in hypotonic Ringer. Hydraulic conductivities were measured on fixed bladders; there was no net water flux across the bladder wall during the hormonal challenge. Values given are the mean and the standard error of the mean on the number of bladders indicated in parentheses.

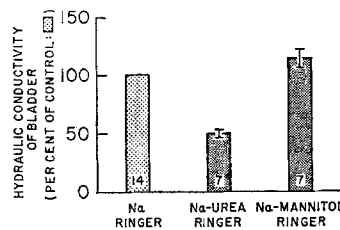


FIGURE 3. Diminished bladder responsiveness to vasotocin (10^{-7} M) in urea-Ringer. One-half the usual NaCl content of Ringer was replaced by equiosmotic concentrations of urea or mannitol during hormonal challenge. Hydraulic conductivities were measured on fixed bladders in full strength NaCl-Ringer. Values given are the mean and the standard error of the mean on the number of bladders indicated in the bars.

response in 55 mM NaCl-Ringer is reduced even more severely (i.e. by about 70% in Fig. 2).

Inhibition of Neurohypophyseal Hormones by Mucosal-to-Serosal Water Flux

Experiments were carried out to test the possibility that net mucosal-to-serosal water flux diminishes the responsiveness of the bladder to oxytocin and to vasotocin. In the study depicted in Table II and in Fig. 4 the velocity of net water flux across the bladder wall was varied by altering the osmotic driving force. This was accomplished by exposing all bladders to identical conditions on the serosal bladder surface, i.e. to 230 mosmoles/kg H₂O Ringer's fluid containing a final concentration of 1×10^{-7} M oxytocin, but exposing the bladder mucosa to 30, 133, or 175 mosmoles/kg H₂O fluid. The velocity of water flux was greatest in bladders which had been filled with the 30 mosmoles/kg H₂O fluid, so that the initial osmotic pressure difference across the bladder wall amounted to 200 mosmoles/kg H₂O. These bladders

TABLE II
HYDROSMOTIC ACTION OF OXYTOCIN AT DIFFERENT OSMOTIC PRESSURE GRADIENTS

Initial serosal (S) and mucosal (M) osmolality	PREFIXATION				POSTFIXATION*		
	Wt. loss/bladder per 30 min				Wt. loss/bladder per 15 min		
	No hormone	10 ⁻⁷ M oxytocin	Mean gradient	Hydraulic conductivity	No hormone	Mean gradient	Hydraulic conductivity
mosmoles/kg H ₂ O	mg	mosmoles/kg H ₂ O	$\mu\text{l} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{mosmoles/kg H}_2\text{O})^{-1} \cdot 10^{-5}$	mg	mosmoles/kg H ₂ O	$\mu\text{l} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \cdot (\text{mosmoles/kg H}_2\text{O})^{-1} \cdot 10^{-5}$	
Bladder A (n=5) S: 230, M: 30	24±9	2393±104	172±3	45.5±2.0	736±52	188±2	25.6±1.8
				<i>P</i> <0.05			<i>P</i> <0.001
Bladder B (n=5) S: 230, M: 133	10±5	1115±12	72±1	50.8±1.3	1453±54	180±2	50.0±3.9
Bladder A (n=5) S: 230, M: 30	28±7	1671±188	173±4	31.5±3.6	496±60	179±2	18.3±2.2
				<i>P</i> >0.5			<i>P</i> <0.01
Bladder B (n=5) S: 230, M: 175	8±4	435±37	50±1	29.0±2.5	960±109	171±3	37.1±4.2

Values given are the mean and the standard error of the mean on the number of experiments indicated in parentheses.

* All fixed bladders were exposed on the serosa to full strength Ringer's fluid (230 mosmoles/kg H₂O) and on the mucosa to Na-free Ringer's fluid (30 mosmoles/kg H₂O).

could not sustain a maximal rate of water flux. In the example shown in Fig. 4 net water movement declined to 60% of maximum 32.5 min after the initial exposure of the bladder to oxytocin in the presence of a 200 mosmoles/kg H₂O gradient. In contrast, bladders which had been subjected to a lesser gradient, i.e. 97 or 55 mosmoles/kg H₂O, could sustain a near maximal rate of water flux for a considerably longer period of time. In order to determine whether the progressive inhibition of net water flux across bladders exposed to the initial gradient of 200 mosmoles/kg H₂O was due to a decrease in the hydraulic conductivity of the bladder wall, bladders were fixed with glutaraldehyde and allowed to equilibrate in Ringer's fluid. Net water flux was then measured across the fixed bladders in the presence of identical osmotic pressure gradients, i.e., bladders were filled with 30 mosmoles/kg H₂O fluid and suspended into 230 mosmoles/H₂O Ringer's fluid. Net water flux was approximately one-half as rapid across fixed bladders which had been exposed to a 200 mosmoles/kg H₂O gradient during the hormonal challenge period than it was across fixed bladders which had been exposed to either a 97 or a 55 mosmoles/kg H₂O gradient during hormonal challenge (Fig. 4). Thus, the

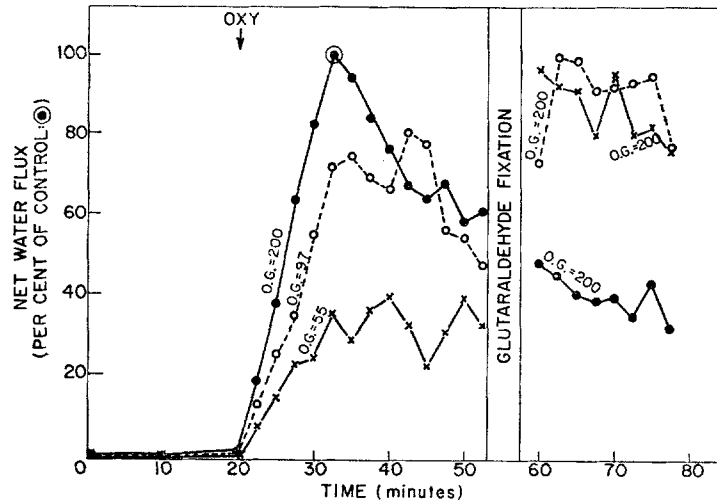


FIGURE 4. Time-course of the hydroosmotic response of bladders to oxytocin (10^{-7} M) at various transmembrane osmotic pressure gradients (O.G.). See Table II.

hydraulic conductivity of bladders exposed to oxytocin and to an initial osmotic pressure gradient of 200 mosmoles/kg H_2O for 30 min and then fixed, is diminished by about 50% (Table II). However, the average hydraulic conductivity of the nonfixed bladder over the entire 30 min interval of exposure to hormone may only be diminished by 10%, or not at all, in the presence of a 200 mosmoles/kg H_2O gradient. This finding has been interpreted to indicate that a diminution in hydraulic conductivity of the bladder wall occurs only in the latter part of the 30 min challenge period, so that the average hydraulic conductivity over a 30 min interval would be greater than the hydraulic conductivity measured at the very end of that interval.

The progressive development of bladder inhibition by high rates of mucosal-to-serosal water flux has been followed over a 30 min interval in Fig. 5. A control set of bladders was challenged with 1×10^{-7} M vasotocin in the absence of an osmotic pressure gradient, i.e., both the serosal and the mucosal solutions were isotonic Ringer's fluid. A contralateral set of bladders from the same toads was exposed to the same serosal hormone bath, but all bladders were filled with 30 mosmoles/kg H_2O Na-free Ringer. Bladders were fixed as usual after 5, 10, 15, or 30 min of exposure to hormone, and the hydraulic conductivities of the fixed bladders were measured in the presence of a 200 mosmoles/kg H_2O gradient. The hydraulic conductivities of both the control and the experimental sets were similar at 5 min of exposure to hormone. However, after 5 min bladders challenged with hormone in the presence of an osmotic gradient became progressively inhibited so that by 30 min the hydraulic conductivity was 40% less ($P < 0.01$) in these bladders than in their controls. Thus, "flux inhibition" is not only a function of the osmotic pressure gradient

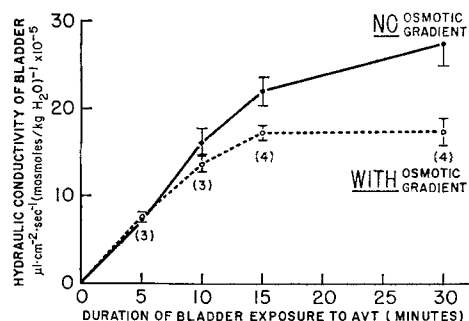


FIGURE 5. Diminished bladder responsiveness to vasotocin (10^{-7} M) in the presence of mucosal-to-serosal water flux. Bladders with osmotic gradient experienced net water flux during hormone challenge before fixation. Hydraulic conductivities were measured on fixed bladders under standard conditions. Values given are the mean and the standard error of the mean on the number of bladders indicated in parentheses.

across the bladder wall, but it is also a function of the duration of bladder exposure to hormone and to osmotic flux.

DISCUSSION

Several decades ago Brunn first showed (5) that frogs gain weight when injected with neurohypophyseal hormones while being kept in water. Similarly, toads which had been injected periodically with vasopressin in the present study absorbed water from their surrounding so that their plasma became diluted from 250 to 158 mosmoles/kg H_2O during a period of 3 days. The experiments in Fig. 1 and Table I have shown that an acute injection of vasopressin is considerably less effective in raising the permeability to water of the urinary bladder of overhydrated toads than of normally hydrated toads. The decline in bladder responsiveness to vasopressin was most dramatic in toads whose plasma was diluted from 250 to 190 mosmoles/kg H_2O . In this range of tonicity the hydraulic conductivity of bladders decreased by about 90% in the *in vivo* studies. In the *in vitro* studies (Fig. 2) the hydraulic conductivity of bladders diminished only by 40% in the same range of tonicity. This difference in response of intact and isolated bladders may have been due to a more intensive stimulation of the isolated bladder with saturating concentrations of vasotocin (8) compared to the barely maximal concentration of injected vasopressin used to stimulate the *in situ* bladder (10). Alternatively, the difference in response could have been due to a lower reactivity of the bladder to hormone while *in situ*. This diminished responsiveness *in situ* could have resulted from a more prolonged exposure of the bladder epithelium to a hypotonic environment in addition to metabolic changes accompanying the hyponatremic state. While the most striking inhibitory effect observed upon administering vasopressin and water to toads over a 3 day period is clearly

due to the overhydration, the possibility that a more intensive or prolonged course of vasopressin injections in normally hydrated toads might also lead to an unresponsive bladder is quite possible. Indeed, it has been observed (10) that the *in vitro* toad bladder becomes completely unresponsive to hormone after having been incubated for 20 hr in isotonic Ringer's fluid containing 1×10^{-7} M vasotocin. This inhibitory phenomenon was not seen *in vivo* when normally hydrated toads were injected with 10 U aqueous Pitressin every 12 hr for 3 days (Table I).

In the present study changes in bladder responsiveness to neurohypophyseal hormones have been based upon changes in the hydraulic conductivity of bladders which had been fixed with glutaraldehyde. The hydraulic conductivity of glutaraldehyde-fixed bladders is not identical to that of nonfixed bladders. In one series of experiments in which bladders had been challenged with a submaximal concentration of oxytocin and then fixed by the same methods employed in this study, the hydraulic conductivity of the bladder after fixation was 22% less than it had been immediately before fixation (10). The possibility cannot be excluded, however, that fixation may have diminished the hydraulic conductivity of bladders by a factor greater or less than 22% when the bladder had been stimulated with higher concentrations of hormone and fixed in a variety of different osmotic environments as was the case in the present study. Moreover, the permeability to water of the fixed bladder is not entirely stable, but it changes as a function of the pre-fixation challenge with hormone, the duration of mucosal-to-serosal water flux across the fixed membrane, and the temperature of the system (9). Furthermore, it has been noted that the hydraulic conductivity of the fixed bladder increases by 30% when 55 mM NaCl-Ringer's fluid (133 mosmoles/kg H₂O) is used as the serosal bathing medium instead of the usual 110 mM NaCl-Ringer's fluid (230 mosmoles/kg H₂O). Therefore, control and experimental hemibladders were always exposed to the same concentration of hormone; they were always fixed with 1% glutaraldehyde in 0.05 M cacodylate buffer and only on the mucosal bladder surface for 5 min; net water flux across the fixed bladders was studied at room temperature and only for the first 15 min after fixation; the hydraulic conductivity was always calculated from the net water movement which occurs when the serosal fluid is 110 mM NaCl-Ringer and the mucosal fluid is 0 mM NaCl Ringer. Under these conditions of experimentation it is suggested that a difference in the hydraulic conductivity of fixed control hemibladders and fixed experimental hemibladders reflects a difference in responsiveness of these bladders to hormone before fixation.

Hays and Leaf (12) have shown that net water flux across the isolated toad bladder diminishes by 43% in the presence of vasopressin when the tonicity of the serosal bathing medium is lowered from 219 to 151 mosmoles/kg H₂O and when the osmotic gradient is maintained at 57 mosmoles/kg H₂O by

diluting the mucosal fluid as well. Similar results were observed in this study under different experimental conditions (Fig. 2). Bladders were exposed to hypotonic serosal media and challenged with vasotocin in the absence of an osmotic gradient so that bulk flow of water across the membrane was avoided at a time when the hormone was inducing a change in the hydraulic conductivity of the membrane. The permeability state of the bladder at the end of a 30 min period of exposure to hormone was estimated by fixing the bladder and by measuring its hydraulic conductivity under the standard conditions mentioned above. This procedure avoids the possible interference of net mucosal-to-serosal water flux with the mechanism of hormone action on membrane permeability to water, a point to be considered in more detail later in the discussion. It should be mentioned in this context, however, that the velocity of water flux in the presence of a 57 mosmoles/kg H₂O gradient employed in the study by Hays and Leaf (12) is probably too low (see Fig. 4 and Table II) to inhibit the action of vasopressin on the membrane.

It is apparent from the experiment in Fig. 3 that the diminished responsiveness of the bladder to hormone in a low NaCl medium is due to the low osmolality of the medium because responsiveness of the bladder to hormone could be restored by adding mannitol to the low NaCl medium in sufficient quantity to raise the osmolality of the solution to isotonicity. Equiosmotic concentrations of urea were only partially effective in restoring bladder responsiveness to hormone. Since urea penetrates the toad bladder epithelium quite readily, particularly in the presence of vasopressin (19), an isotonic urea solution is probably less effective than an isotonic mannitol or NaCl solution in preventing water from accumulating in the epithelium. Why a decrease in the effective osmotic pressure of the extracellular fluids diminishes bladder responsiveness to neurohypophyseal hormones, is not clear. Responsiveness of the bladder to hormone presumably depends upon the ease with which hormone can interact with receptors on the basal-lateral plasma membrane of epithelial cells and upon the efficiency with which the hormone-receptor interaction is translated into a structural rearrangement of the apical plasma membrane. Thus, it is felt at present, that cyclic 3',5'-adenosine monophosphate is generated at the basal-lateral membrane by an action of neurohypophyseal hormones on adenylyl cyclase (20). The cyclic nucleotide is thought to activate a protein kinase (15, 16), which, in turn, may trigger a further series of reactions leading to an increase in the number of aqueous channels in a rate-limiting barrier (14) located at, or near, the mucosal epithelial cell surface (21). One explanation for the inhibitory phenomenon in hypotonic media would be that water enters the hormone target cell as the effective osmotic pressure in the interstitial fluid compartment falls below the effective osmotic pressure in the target cell. This would dilute intracellular cyclic 3',5'-adenosine monophosphate and other intermediates involved in

the cellular mechanism of action of neurohypophyseal hormones. Moreover, if the epithelium swells, the receptors on the lateral plasma membranes could be less accessible to hormone, or stretching of the apical plasma membrane could interfere with the final effector process of the hormone.

We have shown earlier (7) that the isolated toad bladder becomes more rapidly resistant to vasopressin in the presence of an osmotic pressure gradient than it does in the absence of a gradient. Those findings are confirmed here with the glutaraldehyde-fixation method. In addition, it has been shown that this "flux inhibition" depends upon the velocity of mucosal-to-serosal water flux. When the velocity of water movement across the bladder was kept low by keeping the osmotic pressure gradient below 97 mosmoles/liter, there was virtually no "flux inhibition" (Fig. 4 and Table II). However, when the velocity of water flux across the bladder was increased by raising the osmotic pressure gradient to 200 mosmoles/kg H_2O , net water flux across the bladder became progressively inhibited. This diminution in net water flux was not due to the formation of unstirred layers in the bladder wall during high rates of water transfer with a consequent reduction in the effective osmotic gradient across the permeability barrier, because when these bladders were fixed with glutaraldehyde and washed, the hydraulic conductivity of these bladders was also diminished. The rate at which bladders became inhibited by high velocities of transmembrane water flux was found to vary between bladders from different toads and, in particular, bladders from different batches of toads. Nonetheless, in all bladders examined there was a substantial reduction in hydraulic conductivity 30 min after their initial exposure to saturating concentrations of hormone in the presence of a 200 mosmoles/kg H_2O gradient. Thus, the relationship between osmotic gradient and net water flux across the hormone-stimulated bladder is not linear as the osmotic gradient approaches 200 mosmoles/kg H_2O . At osmotic gradients below 97 mosmoles/kg H_2O the relationship between net flux and osmotic gradient appears to be linear confirming previous observations by Hays and Leaf (13).

It is possible that the inhibitory phenomenon observed at high velocities of water movement across the bladder is due to a similar mechanism responsible for the inhibition seen with a diminished effective osmotic pressure of the interstitial fluids. As net water flux across the mucosal epithelium exceeds a critical velocity one would expect the interstitial fluids to become diluted below the plasma or serosal fluid medium. Moreover, as water entry into the hormone target cell across the apical plasma membrane exceeds water exit across the basal-lateral plasma membrane, the intracellular volume would increase. Epithelial cell swelling has been reported by Peachey and Rasmussen (21) under these particular circumstances. Target cell dilution, mechanical changes in the epithelium, or simply flushing out of hormone from the intracellular channels are obvious explanations that first come to mind to account for the

“flux inhibition.” Whatever the underlying cause for this phenomenon, its magnitude apparently depends upon (a) the hydraulic conductivity of the apical plasma membrane, (b) the osmotic gradient across the membrane, and (c) the duration of water flux through the membrane.

Since the classical studies of Verney (22), regulation of the osmotic composition of body fluids has been largely ascribed to hypothalamic osmoreceptors which trigger the release of antidiuretic hormone from the neurohypophysis when the plasma becomes concentrated. In the toad, the osmotic threshold for antidiuretic hormone secretion is at approximately 300 mosmoles/kg H₂O (10). As plasma osmolality rises above this threshold water reabsorption from the urine depends primarily upon the circulating level of hormone. As plasma osmolality falls below 200 mosmoles/kg H₂O, the present study has suggested that the bladder becomes virtually unresponsive, even to saturating concentrations of hormone. Thus, when the plasma is dilute and water intoxication is threatened, water reabsorption from the urine is regulated primarily by the level of responsiveness of the hormone target tissue. This peripheral mechanism for controlling water reabsorption from the urine could play an important role in preventing overhydration when antidiuretic hormone is secreted from the neurohypophysis for reasons other than central osmoreceptor activation. There are a number of physiological (11) and pathological (1) conditions in man which are accompanied by high circulating levels of vasopressin, although the plasma osmolality is well below its osmotic threshold for vasopressin secretion. Whether, and under which circumstances, the toad also experiences “inappropriate” secretion of vasotocin are not known. More important is the unanswered question of whether the target organ resistance to vasopressin observed in this study in overhydrated toads is related to the phenomenon of vasopressin “escape” seen in chronically overhydrated mammals (6, 17, 18).

The author wishes to thank Dr. Irving L. Schwartz for helpful discussions and Dr. Roderich Walter for the 8-arginine-vasotocin and oxytocin used in this study.

This work was done during the tenure of an Established Investigatorship of the American Heart Association and supported by United States Public Health Service Grant AM 15622 of the National Institute of Arthritis and Metabolic Diseases.

Received for publication 19 May 1972.

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