Urea transport across urinary bladder and salt acclimation in toad (*Bufo viridis*)

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SHPUN, S., AND U. KATZ. *Urea transport across urinary bladder and salt acclimation in toad (Bufo viridis).* Am. J. Physiol. 258 (Regulatory Integrative Comp. Physiol. 27): R883–R888, 1990.—The fluxes of urea across the urinary bladder of the toad *Bufo viridis* have been studied under conditions of acclimation to tap water or 500 mosM NaCl solution. The [^{14}C]urea fluxes were measured simultaneously with [^{3}H]inulin to test for nonspecific leakage. The fluxes are quite high (K_{trans} = 75 × 10^{-7} cm/s at 5 mmol/l urea) and are similar in either the mucosal-to-serosal or the opposite direction. "Summer" rates were five to six times higher than the "winter" rates. Antidiuretic hormone (ADH), theophylline, and forskolin increased the fluxes to variable degrees (two to five times), similar in the two acclimation conditions. Phloretin inhibited the urea fluxes by nearly 50%. 1,3-Dimethylurea and thiourea, but not acetamide, competed with the urea fluxes effectively. The fluxes of urea were not affected by the osmotic water flow, although both responded to ADH. It is concluded that urea transport across the urinary bladder of *B. viridis* is by facilitated diffusion through a specific pathway independent of water flux. The inhibitory effect of the structural analogues on the urea flux was affected by salt acclimation, whereas most other characteristics did not differ significantly at 5 mmol/l external urea under the two conditions of acclimation.

antidiuretic hormone; theophylline; forskolin; phloretin

THE EURYHALINE TOAD (*Bufo viridis*) can be readily adapted to hyperosmotic NaCl solutions (10, 23, 25). Adaptation is accompanied by a large increase in the plasma osmolarity, mainly through urea accumulation. An inducible active transport of urea across the skin, which was studied in vitro under salt acclimation (8, 13), could help to prevent urea loss. In vivo studies have suggested also that the kidneys and the urinary bladder play an important role in urea retention in *B. viridis*, particularly in the earlier stages of salt acclimation (12). There is no information yet, however, on the urea transport across the urinary bladder of this species. Urea movement across the urinary bladder of *Bufo marinus* (15, 19) and *Bufo bufo* (1), which was studied quite extensively, proceeds through a mechanism of facilitated diffusion (22) and is greatly increased by antidiuretic hormone (ADH).

We have recently demonstrated that urea flux across the urinary bladder of *B. viridis* saturates at an external urea concentration of ~150 mmol/l (24) and suggested that it could also be described in terms of a facilitated diffusion mechanism. In this paper, we further characterize the urea transport pathway across the urinary bladder of *B. viridis* using ADH, phloretin, and structural analogues of urea, both under tap water and salt-acclimation conditions.

MATERIALS AND METHODS

Isolated urinary bladders of *B. viridis* were used. Animals (30–45 g) were kept in plastic containers and had free access to tap water. Experimental animals were kept for at least 10 days in either tap water or 500 mosM NaCl ("500") solutions, which were changed daily. Adaptation was achieved gradually, by keeping the toads in 2 cm 230 mosM NaCl for 2–3 days before their transfer into 500 mosM NaCl solution. The animals were not fed during the experimental period.

Bladders (average volume was 10 ml) were mounted between two halves of lucite chambers (aperture 1.0 cm^2; hemichamber volume 5 ml) as detailed elsewhere (24). The normal Ringer solution that bathed both sides of the tissue contained (in mM) 112 NaCl, 5 KCl, 1 $CaCl_2$, 2.5 $NaHCO_3$, 5 urea, and 30 mannitol (pH = 8). Total osmolarity of the normal Ringer was 277 mosM, and 230 mosM mannitol was added to the solution used for the bladders of the 500 toads. The solutions were aerated at room temperature (19–23°C).

[^{14}C]urea (Sigma; specific activity 9 mCi/mmol; final concentration 1 μCi/mmol) and [^{3}H]inulin (NEN; specific activity 140.9 mCi/g) fluxes were measured simultaneously. The radioactive tracers were added to one side ("hot"), of the tissue and equilibrated for 60 min; the "cold" side was then replaced every 20 min. Samples of 0.1 ml from the latter side were added to 3.0 ml of scintillation cocktail and counted by a Beckman 9800 spectrometer (corrected for quenching).

Urea permeability and fluxes were calculated using the following equations

$$K_{trans}\ (cm/s) = \frac{cpm \times volume}{cpm^* \times A \times time(h) \times 3{,}600} \quad (1)$$

where K_{trans} is the permeability coefficient, cpm (counts/min) is radioactivity on the cold side, cpm* is radioactivity on the hot side, volume is volume of the cold chamber (cm^3), A is surface area (cm^2), and 3,600 is for converting hours to seconds.

For the calculation of the fluxes, the urea concentration is added

$$flux\ (\mu mol \cdot h^{-1} \cdot cm^{-2}) = \frac{cpm \times [C]_{urea}}{cpm^* \times A \times time(h)} \quad (2)$$

where $[C]_{urea}$ is concentration of urea on the cold side.

The transported urea was checked in a few experiments by the method of Garcia-Romeu et al. (8). Briefly, samples of solutions (1.5 ml) from both sides of the bladder were incubated with urease (1 mg/ml) at 37°C for 40 min and bubbled with a 5% CO_2-95% O_2 mixture to accelerate $^{14}CO_2$ removal. Addition of 8 N HCl and regasification for 30 min eliminated the possibility of carbonate precipitation. The samples were then counted. The test verified that the urea moved intact across the wall of the urinary bladder.

The experiments where water movement was determined together with the urea flux were carried out on bladder sacs that were tied on a glass tube. Osmotic water flow was followed gravimetrically (3), with the inside of the bladder (mucosal side) filled with Ringer solution diluted with distilled water. The bladder was drained gently on the smooth edge of a glass beaker and was weighed every 20 min to the nearest 0.01 g. The surface area of the sacs was calculated, assuming the two lobes of the bladder as two spheres and estimating their radius from the volume.

ADH (lysine vasopressin or oxytocin), theophylline, forskolin, and phloretin were from Sigma; 2,3-dinitrophenol (DNP) was from Merck.

Statistical significance was analyzed using Student's *t* test. For multiple analyses, analysis of variance (ANOVA) was used.

RESULTS

Nearly 120 urinary bladders are included in this study. Table 1 shows the fluxes and K_{trans} of both urea and inulin, which were measured simultaneously on the same preparations. Very small fluxes of inulin were measured across the bladders under our experimental conditions; its K_{trans} ($\sim 10 \times 10^{-7}$ cm/s) is only four times higher than in the sac preparation ($2.4 \pm 0.8 \times 10^{-7}$ cm/s, $n = 6$), showing that our preparations did not suffer much from edge damage. When the fluxes that were carried out during a period of over 2 yr were summarized, two groups could be distinguished with respect to urea fluxes: "winter" rates (December to February) as compared with "summer" rates (May to July). Despite the somewhat high base-line permeability, the seasonal difference in the urea transport seems quite specific, presumably cellular, since the K_{trans} of inulin, which is considered extracellular, was the same throughout. Although the seasonal effect was not investigated systematically, it should not affect our results because two experimental groups were always run together. At 5 mmol/l external urea, the fluxes were a little higher in the tap-water group (Table 1 and Fig. 1), but the difference was not statistically significant. The increased flux in the salt-acclimated group is seen only at higher urea concentrations (24).

Fluxes in the mucosal-to-serosal (m → s) and serosal-to-mucosal (s → m) directions. Fluxes in the two directions were measured on the same pieces of urinary bladder. The chambers were washed three to five times before measurements in the opposite direction began. Identical solutions bathed the tissue on both sides, and urea concentration was 5 mmol/l. Figure 1 shows that K_{trans} (permeability coefficient) did not differ significantly in either of the directions under both conditions of acclimation. Similar results have been reported in the literature (15, 19).

Effect of DNP. The effect of DNP, an uncoupler of oxidative phosphorylation that reduces the level of cellular ATP, was tested on bladders from the two conditions of acclimation. The tissues were incubated with DNP (2×10^{-4} M, dissolved in ethanol) in the serosal bath for 30 min. Urea concentration was 5 mmol/l. The fluxes of urea (m → s) with and without DNP were 167 ± 47 $\mu mol \cdot h^{-1} \cdot cm^{-2}$ and 183 ± 83 $\mu mol \cdot h^{-1} \cdot cm^{-2}$, respectively, in the tap water group and 130 ± 39 $\mu mol \cdot h^{-1} \cdot cm^{-2}$ and 121 ± 46 $\mu mol \cdot h^{-1} \cdot cm^{-2}$, respectively, in the 500 mosM group (six preparations under each condition; summer rates). None of the fluxes differed significantly from any of the other (ANOVA). These results show that the urea flux is independent of oxidative metabolism and suggest passive movement.

Effect of sodium on urea fluxes. The effect of sodium on the urea fluxes was studied by replacing all the NaCl in the Ringer solution by either mannitol or choline chloride. Table 2 shows that the K_{trans} of urea, which was calculated for the m → s direction, was independent of the presence of sodium. None of the differences was statistically significant.

Effect of ADH, theophylline, and forskolin. The effect of vasopressin (50 mU/ml in the serosal bath) on the urea flux was measured in the m → s direction at a urea concentration of 5 mmol/l in bladders from toads of the two acclimation conditions. The effect of ADH was quite variable; no effect was seen on the permeability in the urinary bladders from the tap-water group, which are

TABLE 1. *Fluxes and K_{trans} of urea and inulin in mucosal-to-serosal direction across flat pieces of isolated urinary bladders in winter and summer toads*

	Winter		Summer	
	Tap water	500 mosM NaCl solution	Tap water	500 mosM NaCl solution
Urea				
Flux, $\times 10^3$ nmol·h^{-1}·cm^{-2}	21.6±8.4 (25)	16.7±9.2 (15)	139±40.9 (49)	131±41.8 (54)
K_{trans}, $\times 10^{-7}$ cm/s	12±4.7	9.3±5.1	77.2±22.6	72.8±23.2
Inulin				
Flux, nmol·h^{-1}·cm^{-2}	4.5±3.2 (10)	3.6±2.2 (8)	3.2±0.8 (49)	3.4±1.2 (54)
K_{trans}, $\times 10^{-7}$ cm/s	13.8±11.9	11.0±6.7	9.8±3.9	10.6±2.4
Ratio of urea/inulin fluxes, $\times 10^3$	4.80	4.64	43.44	38.53

Values are means ± SD; no. bladders in parentheses. Bath concentrations: urea 5 mmol/l and inulin 1 μmol/l. K_{trans}, permeability coefficient.

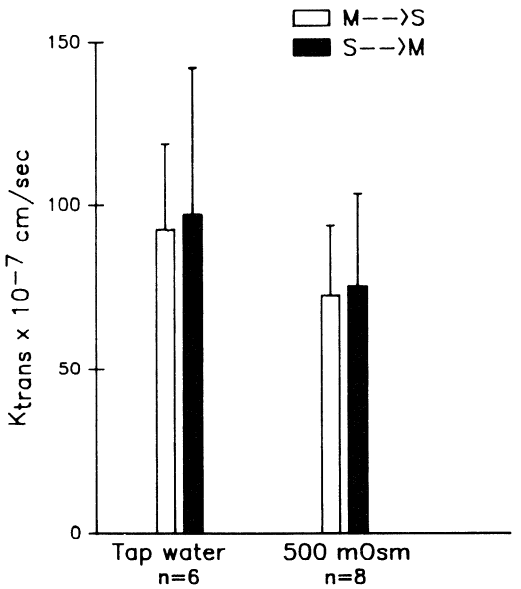

FIG. 1. K_{trans} of bidirectional urea fluxes across the urinary bladder of tap water and 500 mosM NaCl acclimated toads (*B. viridis*). (M, mucosa; S, serosa). Bar is 1 SD; *n*, no. bladders.

TABLE 2. *Effect of sodium replacement on K_{trans} of urea in mucosal-to-serosal direction*

	Tap Water	500 mosM NaCl Solution
Control*	108.6±38.8 (6)	66.4±12.3 (7)
Mannitol replacement	74.9±29.1	69.4±10.0
Control†	11.2±7.6 (4)	
Choline chloride replacement	14.3±9.0	

Values are means ± SD; no. bladders in parentheses. K_{trans}, permeability coefficient ($\times 10^{-7}$ cm/s). * Summer measurements; † winter measurements.

TABLE 3. *Effect of vasopressin and theophylline on K_{trans} of urea (mucosal-to-serosal direction) in summer toads*

	Tap Water	500 mosM NaCl Solution
Control	70.6±34.4 (6)	72.2±32.8 (6)
Vasopressin, 50 mU/ml	73.9±18.9	94.4±41.6*
Control	108.6±38.8 (6)	66.4±12.3 (7)
Theophylline		
1 mmol/l	139.0±51.7*	84.7±14.7*
5 mmol/l	237.2±67.6*	110.8±24.8*

Values are means ± SD; no. bladders in parentheses. K_{trans}, permeability coefficient ($\times 10^{-7}$ cm/s). * Significantly different at $P < 0.05$, paired *t* test.

summarized in Table 3 (summer rates). In the 500 mosM NaCl-acclimated group the permeability increased, however, by ~30% ($P < 0.013$; paired *t* test). On the other hand, the fluxes that are summarized in Fig. 2 (tap-water acclimation) were nearly doubled on the application of oxytocin. The response to ADH did not correlate with the basal rate of urea flux (statistical analysis showed that the slope of the regression line relating the ADH response to the basal rate of urea flux was close to zero, with large scatter of the observed points). No effect of the hormones was seen on the fluxes of inulin.

Because vasopressin is known to increase intracellular

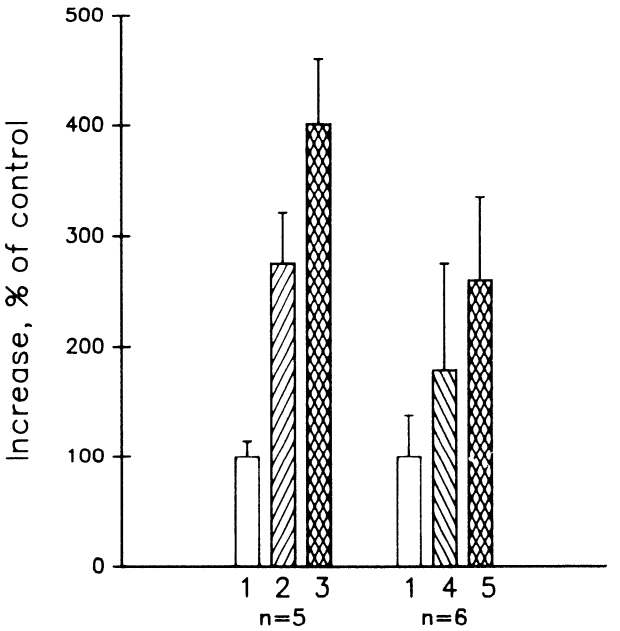

FIG. 2. Additive increase of urea flux on application of oxytocin and theophylline (*1*, control; *2*, oxytocin; 100 mU/ml; *3*, oxytocin plus theophylline, 5 mmol/l; *4*, theophylline; *5*, theophylline plus oxytocin). Bar, 1 SD; *n*, no. bladders.

adenosine 3′,5′-cyclic monophosphate (cAMP), the second messenger for the action of the hormone (17), we have tried the effect of theophylline, which inhibits the phosphodiesterase, thus increasing the cellular cAMP. Theophylline (1 or 5 mmol/l) was added to the serosal side, and urea flux was measured at a bath concentration of 5 mmol/l on both sides. The results show (Table 3) that theophylline at 1 mmol/l increased the permeabilities similarly in the two groups of acclimation [23% ($P < 0.05$) in the tap-water and 24% ($P < 0.001$) in the 500 group; paired *t* test]. A greater effect was observed with 5 mmol/l theophylline. These results suggest that the flux of urea across the bladder is sensitive to cAMP. In another set of experiments, we tested the additive effect of oxytocin and theophylline on bladders from tap-water acclimated toads (Fig. 2). The order of addition of the drugs was alternated on the various preparations. Although the stimulation was additive, the degree of stimulation depended on the sequence of addition.

Forskolin, which also elevates cellular cAMP through direct interaction with the catalytic subunit of the adenylate cyclase (21), was applied at 4 μmol/l and was effective when applied either from the serosal or mucosal side. It was more potent than the previous agents and increased the m → s urea flux three times and more (43.4 ± 17.1 before and 120.9 ±19.4 $\mu mol \cdot h^{-1} \cdot cm^{-2}$ after forskolin in five winter bladders at 5 mM urea concentration), both in the tap water and the salt-acclimated groups.

Effect of phloretin. Phloretin is known as an inhibitor of urea transport across amphibian urinary bladder (15). The drug (5×10^{-4} M) was added to either the mucosal or serosal side, and urea fluxes were then measured at a bath concentration of 5 mmol/l. Only the flux in the m → s direction was affected by phloretin, decreasing it nearly 50% (Fig. 3; $P < 0.016$), irrespective of the side to

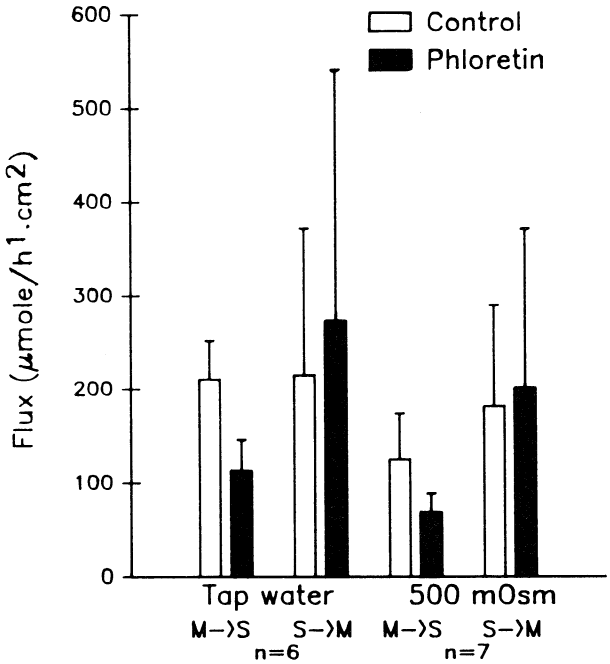

FIG. 3. Effect of phloretin (5×10^{-4}) on bidirectional urea fluxes across urinary bladder of *B. viridis*, both under tap-water and salt acclimation conditions. Phloretin was added to mucosal (M) side. Bar, 1 SD; S, serosal; *n*, no. bladders.

which the drug was added. It was effective on bladders from both conditions of acclimation. Ethanol, which was used to dissolve the phloretin (and DNP) at a final concentration of 0.5%, did not affect the urea fluxes significantly.

The effect of structural analogues of urea. Three structural analogues (1,3-dimethylurea, thiourea, and acetamide) were used, and their effect on the urea flux was tested in both directions. They were added at concentrations of 20 mmol/l to the side of the bladder from which the flux was measured (in the presence of 5 mmol/l urea). The order in which the analogues were tested was randomly changed in every experiment, and the preparations were washed three to five times before testing the next analogue. Figure 4 summarizes the inhibitory effect of the analogues on the fluxes of urea under the two conditions of acclimation. 1,3-Dimethylurea had the largest effect. The degree of inhibition between the two groups of acclimation was significantly different (71% in the tap-water and 50% in the 500 group; $P = 0.036$). Thiourea was less effective in both groups; the urea flux decreased 38% in the tap-water group and 25% in the 500 group. Acetamide did not have a significant effect on the urea fluxes in either group of acclimation. Control fluxes (urea alone), which were carried out between periods of the tested analogues, were not affected significantly (ANOVA), indicating that the effect of the analogues was completely reversible.

Interaction of urea and water fluxes. To test the effect of osmotic water flow, measurements were made simultaneously with the urea flux. Water movement was determined gravimetrically on sac preparations with 100 mosM osmotic gradient (diluted inside), while the flux of urea in the m → s direction was measured at 5 mmol/l on both sides using [^{14}C]urea. Figure 5 shows a typical

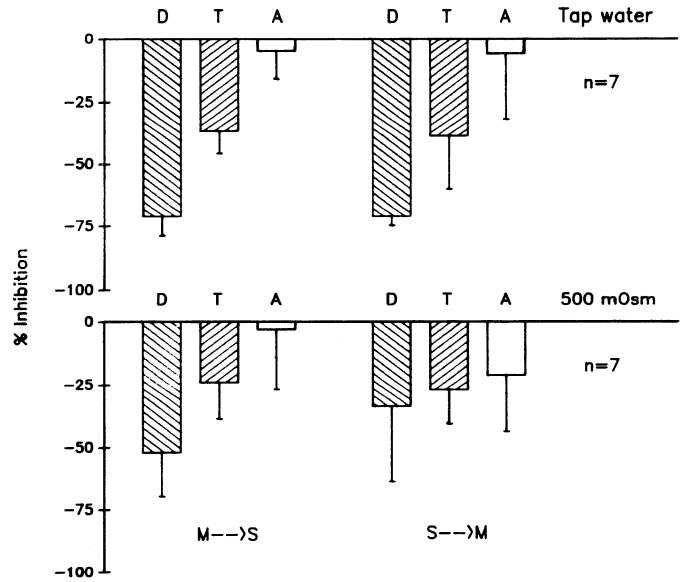

FIG. 4. Inhibitory effect of three structural analogues of urea (D, dimethylurea; T, thiourea; A, acetamide), on bidirectional urea fluxes under two conditions of acclimation. Concentration of analogues was 20 mmol/l at urea concentration of 5 mmol/l. Bar, 1 SD; S, serosal; M, mucosal; *n*, no. bladders.

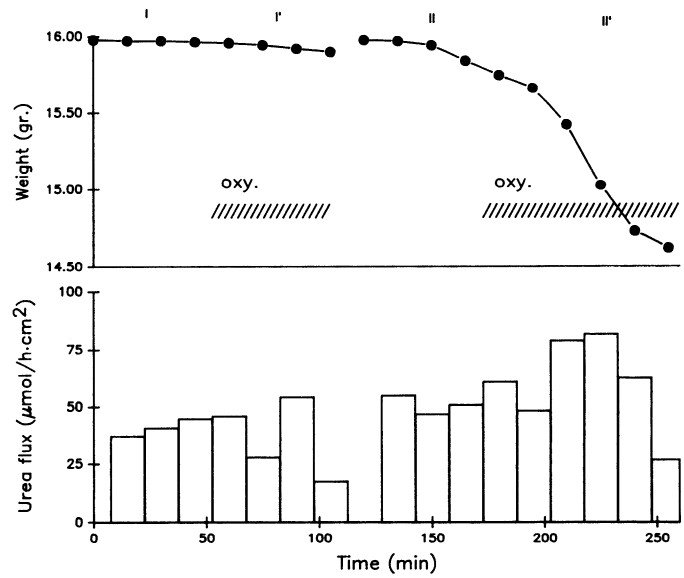

FIG. 5. Typical experiment where mucosal-to-serosal urea flux and osmotic water flow were measured simultaneously on same urinary bladder. I, identical solutions on both sides. II, 100 mosM osmotic gradient (diluted on mucosal side). Oxy, periods in presence of 50 mU/ml oxytocin (I′, II′).

experiment, and Table 4 summarizes the results. Water movement that was negligible under osmotic gradient (period II in Fig. 5) was greatly enhanced on the application of oxytocin (period II′). The hormone elevated the flux of urea, but the increase was quite similar in the presence or absence of the osmotic gradient (periods I′ and II′ in Fig. 5 and Table 4). This result supports the conclusion of Carvounis et al. (4) about the independence of the water and urea pathways.

DISCUSSION

The experiments that are described in this paper confirm the results of our previous report (24). They show

TABLE 4. *Water and urea fluxes in urinary bladders from tap-water acclimated summer toads measured on sac preparations*

	Control (No Gradient)			100 mosM Osmotic Gradient		
	Control	Oxytocin	*P*	Control	Oxytocin	*P*
Water, $mg \cdot h^{-1} \cdot cm^{-2}$ (6)	1.1±0.6	2.8±1.5	<0.05	7.4±4.7	39.9±14.8	<0.01
Urea, $\mu mol \cdot h^{-1} \cdot cm^{-2}$ (6)	52.4±23.0	128.3±88.2	NS	68.7±18.9	138.0±66.9	<0.05

Values are means ± SD; paired *t* test; no. bladders in parentheses. Urea concentration was 5 mmol/l, oxytocin was 50 mU/ml. NS, not significant.

that the urea transport across the urinary bladder of *B. viridis* conforms with the definitions of facilitated diffusion (22) and is saturable. Long-term acclimation in hyperosmotic NaCl solution seems to involve a change in the maximum velocity (V_{max}) rather than the Michaelis constant (K_m) of the pathway (24); most other characteristics do not seem to show significant differences under these conditions. A twofold increase in the K_{trans} of urea was found also in *B. marinus* urinary bladder on saline acclimation (J. S. Handler, personal communication). The difference between the two conditions of acclimation is apparent, however, only at higher bath urea concentrations (24); at the concentrations used in the present study (5 mmol/l) the fluxes do not differ significantly between the two groups. Other characteristics, such as the responses to theophylline and forskolin and sensitivity to phloretin, did not seem to differ significantly under the two conditions of acclimation; they differed though in their sensitivity to the structural analogues. Earlier experiments on the transport of sodium across the urinary bladder of this species have similarly shown that it was also not affected by salt acclimation (11). On the other hand, both the sodium and urea transport across the skin of this toad have been markedly affected by salt acclimation (11, 13).

The reported K_{trans} of urea fluxes across urinary bladders in the literature range between 12 and 170 × 10^{-7} cm/s (5, 7), and our values fall within this range. The measured permeability coefficient of *B. marinus* urinary bladder to urea, under the same experimental conditions in our laboratory (unpublished observations), was indeed much lower than the permeability of the bladder of *B. viridis* (0.3 vs. 30 × 10^{-7} cm/s, at a urea concentration of 5 mmol/l). The large variability in the urea fluxes across toad urinary bladders, which depends on the season (6), may also depend on some unidentified hormonal factor(s). The response to ADH was clearly highly variable, whereas theophylline and forskolin were much more effective. Because we used >50 mU/ml of the various hormones (oxytocin, lysine vasopressin, and also arginine vasotocin), it suggests that all the hormones we used were not specific enough for the receptor.

Although the m → s fluxes of urea did not differ significantly from the fluxes in the opposite direction under control conditions, it is interesting to note that phloretin affected the fluxes only in the m → s direction regardless of the side of its addition. It indicates the vectorial nature of this pathway, where the mucosal side seems to be the limiting step (18).

The urea transport across the urinary bladder of *B. viridis* saturates at urea bath concentrations of >150 mmol/l (24); this rather high value can be related to the high concentrations found in the plasma of this species under various environmental conditions (9, 10). In the studies made with structural analogues on two other species (14, 16, 19), it is hard to discern a systematic order in their effect on the urea flux. In our experiments, the two compounds that have symmetrical structure, i.e., dimethylurea and thiourea, exerted stronger competition with the fluxes of urea. The latter is also symmetrical. It will be interesting to explore this possibility further. The structural analogues were tested at 5 mmol/l urea bath concentration. In the light of the high K_m of the transport system (24), it might also be revealing to test the effects of the analogues at urea bath concentrations closer to the physiological values.

The urea transport pathways across the skin and the urinary bladder are quite different from each other. The apparent K_m for urea is very different in the two epithelia (2 mmol/l in the skin and >150 mmol/l in the bladder), and only in the bladder but not the skin the rate of urea flux increases in response to the application of ADH and theophylline. Moreover, urea transport across the skin is against a concentration gradient, i.e., an energy-dependent active process (8, 13, 20), whereas in the urinary bladder the mechanism resembles facilitated diffusion. Common to both epithelia is the inhibition by the specific drug phloretin and the stimulation in response to the application of forskolin (see Ref. 1; unpublished data). The functional significance of the differences between the two epithelia could be reconciled when the osmoregulatory roles that are played by each epithelium in the anuran amphibians are considered: the skin provides the contact with the external environment, whereas the urinary bladder participates importantly in the regulation of the composition of the body fluids (12). Accordingly, the urea permeability of the skin is rather low (8, 13), and the induced urea influx on salt acclimation would aid to prevent the loss of urea through its reuptake across the integument. On the other hand, the enhanced recycling across the urinary bladder is fulfilled by a high diffusive urea permeability, which increases on salt acclimation.

In conclusion, we have verified that the urea transport system across the urinary bladder of the toad *B. viridis* is by a facilitated diffusion mechanism, as it is in the bladders of other anurans. The increase in the urea flux on high salt acclimation that is seen only at high external urea concentrations is due mainly to an increase in the V_{max} of the system (24). It could suggest an increase in the number of the urea transport sites. Of the other characteristics of the system, the inhibitory action of the

analogues was smaller on salt acclimation, whereas the other characteristics did not show significant differences in response to high-salt acclimation conditions.

Dr. J. Haichenco gave helpful assistance in the osmotic water experiments.

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Received 25 April 1989; accepted in final form 20 November 1989.

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