The Journal of Experimental Biology 203, 1027–1037 (2000)
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JEB2442

DIFFERENTIAL BRANCHIAL AND RENAL HANDLING OF UREA, ACETAMIDE AND THIOUREA IN THE GULF TOADFISH *OPSANUS BETA*: EVIDENCE FOR TWO TRANSPORTERS

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Accepted 1 December 1999; published on WWW 21 February 2000

Summary

The possible presence of a urea transporter in the kidney of the gulf toadfish (*Opsanus beta*) and further characterization of the pulsatile facilitated transporter previously identified in its gills were investigated by comparing the extra-renal and renal handling of two urea analogues with the handling of urea. Toadfish were fitted with caudal artery and indwelling urinary ureteral catheters and injected with an iso-osmotic dose of ^{14}C-labelled urea analogue (acetamide or thiourea) calculated to bring plasma analogue concentrations close to plasma urea concentrations. Branchial permeabilities to urea, acetamide and thiourea were similar during non-pulsing periods and all increased during pulse events, although urea permeability was greater than analogue permeability during pulses. The incidence and magnitude of acetamide and urea pulses at the gills were significantly correlated, acetamide pulses being 35–50 % of the size of urea pulses. However, the thiourea and urea pulses at the gills were only weakly correlated, thiourea pulses being less than 16 % of the size of urea pulses. Thiourea inhibited branchial urea excretion by reducing the pulse frequency. The renal handling of thiourea and urea were similar in that both substances were more concentrated in the urine than in the plasma, whereas acetamide was found in equal concentrations in the urine and plasma. Urea and thiourea were secreted 2–3 times more effectively than Cl^- and water, whereas acetamide was secreted at a similar relative rate. The differential handling of the urea analogues by the gills and kidney indicates the presence of a different, possibly unique, transporter in the kidney. The movement of thiourea and urea into the renal tubule against an apparent concentration gradient suggests the presence of an active transport mechanism.

Key words: gulf toadfish, *Opsanus beta*, nitrogen handling, kidney, gill, urea, acetamide, thiourea, transport.

Introduction

Classically, urea was believed to move passively through lipoprotein cell membranes. Over the past few decades, this prevailing doctrine has been refuted by the discovery of various urea transport mechanisms, including facilitated and active carrier-mediated transport. Urea analogues such as thiourea (H_2NCSNH_2) and acetamide (H_3CCONH_2) are useful tools for determining the presence of a transport mechanism. Since their structure is similar to that of urea (H_2NCONH_2), thiourea and acetamide interact competitively at transport sites, thereby inhibiting urea transport (Chou and Knepper, 1989; Gillan and Sands, 1993).

Despite the inhibitory effects, analogues are not always transported as effectively as urea on account of the specificity of the carrier. The vasopressin (arginine vasopressin; AVP)-sensitive facilitated diffusion carrier (originally considered to be UT-2 but now considered to be UT-A1; You et al., 1993; Smith et al., 1995; Shayakul et al., 1996; Karakashian et al., 1999) is characteristically difficult to saturate, even at urea concentrations well beyond the physiological range (Chou et al., 1990). However, urea transporters can often be saturated at substantially lower thiourea concentrations, demonstrating that the transport capacity is much lower for thiourea than for urea (Brahm, 1983; Mayrand and Levitt, 1983; Chou et al., 1990).

Studies involving the characterization of renal urea transport mechanisms have shown not only that transporters move analogues less effectively than urea but that they can often distinguish between analogues. In the kidney of the spiny dogfish shark *Squalus acanthias*, the urea transporter, which reabsorbs urea by apparent primary or secondary active transport, also reabsorbed acetamide, albeit to a lesser extent than urea. However, thiourea was not reabsorbed (Schmidt-Nielsen and Rabinowitz, 1964). Very recently, Smith and

Wright (1999) have cloned a urea transporter from shark (*S. acanthias*) kidney which appears to be related to the mammalian UT-A family of facilitated diffusion transporters, but its role in urea reabsorption remains unknown. In contrast to shark kidney, a study by Schmidt-Nielsen and Shrauger (1963) on the kidney of the frog *Rana catesbeiana* demonstrated the apparent active secretion of thiourea and the lack of acetamide transport. In mammalian studies, it has been demonstrated that thiourea has the ability to pass through the vasopressin-sensitive urea transport mechanism in rat inner medullary collecting ducts, although the permeability of thiourea was much lower than the permeability of urea (Chou et al., 1990). Naccache and Sha'afi (1973) reported human erythrocyte permeability for urea to be 2.4×10^{-5} cm s^{-1} while it was only 0.07×10^{-5} cm s^{-1} for thiourea.

Only recently has investigation into the existence of urea transport mechanisms been initiated in teleost fish. The gulf toadfish *Opsanus beta*, among the best studied in this regard, is one of only a few adult teleosts with an active ornithine–urea cycle (for others, refer to Saha and Ratha, 1987; Randall et al., 1989; Wood et al., 1989). It also has the remarkable ability to switch from ammoniotelism to ureotelism when placed under stressful circumstances such as ammonia exposure, air exposure, crowding and/or confinement (Walsh et al., 1990, 1994; Walsh and Milligan, 1995). The primary route of urea excretion is *via* the gills (>90 % total urea excretion), where excretion is not continuous but occurs in distinct 0.5–3 h pulses, on average once or twice every 24 h (Wood et al., 1995, 1997). Recently, the cDNA for a hormonally controlled facilitated diffusion mechanism related to the mammalian UT-A urea transporters has been isolated in the gills (Smith et al., 1998). The toadfish transporter appears to be functional only when plasma cortisol concentration is low (Walsh, 1997; Wood et al., 1997, 1998) and may be specifically activated by arginine vasotocin (AVT, the piscine analogue of mammalian AVP; Gilmour et al., 1998; Perry et al., 1998). During pulsing periods, the activation of this transporter gives rise to at least a 35-fold increase in urea permeability (Wood et al., 1998). At the same time, there is only a fivefold increase in thiourea permeability during pulses, significantly lower than the increase observed in urea permeability (Wood et al., 1998).

A secondary route of excretion is the kidney (<10 % total urea excretion). The closely related oyster toadfish (*Opsanus tau*) is generally believed to have an aglomerular kidney, although indirect evidence provided by Lahlou et al. (1969) suggested minimal glomerular filtration (Marshall, 1929; Hickman and Trump, 1969). Regardless, urine is formed primarily by secretion. Although minor, the kidney may play a role in the regulation and maintenance of blood urea concentrations. Toadfish urine urea concentrations generally exceed plasma levels by at least 30 %, and both urine and plasma concentrations increase when the fish activate ureotelism (Wood et al., 1995). Thus, the movement of urea into the kidney tubule may occur against a concentration gradient, suggesting the presence of an active transport mechanism.

There were two main objectives of the present study. The first objective was to compare quantitatively the handling of urea and two urea analogues, acetamide and thiourea, by the branchial hormonally controlled facilitated diffusion carrier. Permeabilities for the three substances during pulsing and non-pulsing periods were determined, and the effects of analogues on pulse frequency and size were monitored. The second goal was to investigate the possibility of a renal urea transporter by characterizing the renal handling of urea relative to analogues and other substance such as Na^+, Cl^- and water. The handling of urea, acetamide and thiourea by each organ was compared.

Our results indicate that there exist two distinct transport mechanisms involved in urea excretion in the gulf toadfish, found in the gill and the kidney, each of which differentially handles urea, acetamide and thiourea.

Materials and methods

Experimental animals

Gulf toadfish (*Opsanus beta* Goode and Bean) were caught by commercial shrimpers in Biscayne Bay, Florida, USA, in November and December 1998. The toadfish (70–300 g) were held in an outdoor tank at the shrimpers' holding facility with running sea water (ambient seasonal conditions) for no longer than 24 h following capture, before being transferred to the laboratory. Fish were treated with Malachite Green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet, Hayward, CA, USA) on the day of transfer to the laboratory to prevent infection by the cilate *Cryptocaryon irritans* (Stoskopf, 1993). Initially, the fish were kept in 50 l Rubbermaid containers with flowing, aerated sea water. Fish were maintained in this relatively crowded environment (>10 fish per tank) to initiate a switch to ureotelism (Walsh et al., 1994a). To ensure full expression of ureotelic behaviour, the fish were transferred in groups of four to plastic tubs (6 l) served with flowing sea water at least 48 h prior to surgery. The temperature in these tanks was 26 °C. Fish were fed weekly throughout the experimental period.

Experimental protocol

Caudal artery and ureteral catheterization were performed simultaneously on fish anaesthetized with MS-222 (0.5 g l^{-1}; Sigma) and wrapped with wet towels. Caudal artery catheters were inserted as described by Wood et al. (1997). The caudal vertebrae were exposed by a 1.5–2.0 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with Clay-Adams PE50 tubing filled with Hanks' saline (Walsh, 1987) and 50 i.u ml^{-1} sodium heparin (Sigma). A heat-flared PE160 sleeve was glued in place with cyanoacrylate tissue cement (Vetbond; 3M Corporation) and sutured at the site of exit to secure the catheter. The wound was treated with oxytetracycline powder, to prevent infection, and sutured securely with 2-0 silk.

The technique for inserting indwelling ureteral catheters was modified from the protocol described by Howe and Gutknecht (1978) and Lahlou et al. (1969). Our goal was to bypass the

urinary bladder, thereby eliminating any reabsorptive/secretory roles of the bladder epithelium so as to examine the function of the kidney alone (for a discussion of this problem, see Baustain et al., 1997). The end of a catheter, made of approximately 60 cm of Clay-Adams PE50 tubing, was filled with distilled water and advanced dorsally 1.5–2.0 cm through the urogenital papilla into the urinary sinus. The catheter was held in place by three 2-0 silk ligatures around the papilla and was attached to the body of the fish by four cutaneous ties. A ventral incision approximately 1.5–2.0 cm in length was then made just anterior to the urogenital papilla. Both urinary bladders were isolated and ligated close to the ureter end with 2-0 silk, hence the storage function of the bladders was bypassed, and the urine drained directly from the ureters into the urinary sinus where the catheter tip was located. Oxytetracycline powder was dusted into the body cavity, and the ventral musculature and skin were then sutured securely. During recovery and experimentation, urine was collected continuously, with the catheter emptying into a vial approximately 3.0 cm below the water level of the box. The urine flow rate (UFR) was determined gravimetrically. Following surgery, fish were kept in individual darkened containers that were continuously aerated, supplied with flowing water (200 ml min^{-1}) and had a minimum volume of 1.5 l.

Following the procedure outlined by McDonald and Wood (1998), [^{3}H]polyethylene glycol (PEG, M_r 4000) was used as a marker for glomerular filtration rate (GFR) to determine whether there were any functional glomeruli in this supposedly aglomerular kidney. PEG 4000 was chosen because it is considered to be a more accurate and conservative indicator of GFR in fish than other commonly used markers (e.g. inulin derivatives) because it undergoes minimal radioautolysis, metabolic breakdown or post-filtration reabsorption across the tubules and bladder (Beyenbach and Kirschner, 1976; Erickson and Gingerich, 1986; Curtis and Wood, 1991). Prior to injection of PEG, the fish were allowed to recover from surgery for at least 12 h, a period during which the patency of the arterial and urinary bladder catheters was confirmed. A dose of 0.185 MBq 100 g^{-1} body mass of [^{3}H]PEG 4000 (New England Nuclear) was injected *via* the caudal arterial catheter followed by an additional 0.3 ml of saline in fish in which both catheters were deemed patent. The [^{3}H]PEG 4000 was then allowed to equilibrate throughout the extracellular space for 12 h before sampling commenced.

The patterns of gill urea excretion, UFR, GFR, urine composition, plasma composition and urinary urea and ion excretion rates of the toadfish were monitored. Two series were performed: five fish were treated with acetamide and five fish were treated with thiourea. Mean masses of fish in the acetamide series were 0.264±0.035 kg (means ± S.E.M.), ranging from 0.133 to 0.328 kg. Mean masses of fish in the thiourea series were 0.365±0.038 kg, ranging from 0.256 to 0.462 kg. Following the [^{3}H]PEG 4000 injection and equilibration period, blood, urine and water samples were taken and water flow to the box was then stopped and the volume set to at least 1.5 l. Thereafter, water samples (5 ml) were taken every 2 h for the remainder of the experiment, the water being changed at 24 h intervals. Blood and urine samples were taken every 12 h, making a total of approximately 30 water samples (at 2 h intervals), six blood samples (at 12 h intervals) and six urine samples (each spanning a 12 h collection period). Twenty-four hours after the [^{3}H]PEG 4000 injection, the fish were injected with a dose of 0.185 MBq 100 g^{-1} body mass of ^{14}C-labelled thiourea or acetamide in 420 μmol 100 g^{-1} body mass of iso-osmotic cold urea analogue (concentration 320 mmol l^{-1}) to render internal analogue concentrations approximately equal to internal urea concentrations. Blood samples were immediately centrifuged (10 000 ***g*** for 2 min). Plasma and urine were stored at −20 °C for later analysis of Na^+, Cl^-, urea, [^{14}C]analogue and [^{3}H]PEG 4000 concentrations. Water samples were analyzed for urea, [^{3}H]PEG 4000 and [^{14}C]analogue only.

To determine the normal composition of bladder urine, separate urine samples were taken directly from the bladder of 72 toadfish and analysed for urea, Na^+ and Cl^- concentration. These fish were part of a separate study and were killed by a rapid blow to the head, opened in the ventral midline and sampled by direct puncture of the exposed bladder with a 22 gauge needle and 1 cm^3 syringe.

Analytical techniques and calculations

Urea concentrations in blood, urine and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in the three media and correction for the presence of thiourea and acetamide. This correction was performed by adding the calculated concentration of analogue present in the urine, plasma or water (see equations 1 and 2) in the same volume as the sample to each point of the standard curve of the urea assay. Any interference with the colorimetric assay by thiourea or acetamide would therefore be automatically accounted for when using the respective standard curve, a point that was validated by urea addition/recovery tests. Ammonia concentrations in the water were measured by the method of Ivancic and Degobbis (1984). Na^+ and Cl^- concentrations in plasma and urine were measured using a Varian 1275 atomic absorption spectrophotometer and a Radiometer CMT10 chloridometer respectively. For measurements of [^{3}H]PEG 4000 and [^{14}C]analogue, blood and urine samples (25 μl plus 1 ml of sea water) or water samples (1 ml) were added to 10 ml of ACS fluor (Amersham) and analyzed by scintillation counting on an LKB Rackbeta 1217 counter using an onboard quench correction program to separate ^{3}H and ^{14}C counts.

The concentration of thiourea or acetamide ([analogue], mmol l^{-1}) in the plasma, urine and water was determined from the specific activity (A, cts min^{-1} μmol^{-1}) of the injected solution and then conversion of the radioactivity found in the samples into concentration (μmol ml^{-1}):

$$A = \mathrm{cpm_{in}}/[\mathrm{analogue}]_{\mathrm{cold}}, \quad (1)$$

$$[\mathrm{analogue}] = \mathrm{cpm_s}/A, \quad (2)$$

where $\mathrm{cpm_{in}}$ is the radioactivity of the injected solution (in $\mathrm{cts\,min^{-1}}$), $\mathrm{cpm_s}$ is the radioactivity in the sample ($\mathrm{cts\,min^{-1}\,ml^{-1}}$) and $[\mathrm{analogue}]_{\mathrm{cold}}$ is the concentration of unlabelled analogue ($\mu\mathrm{mol\,ml^{-1}}$). The branchial clearance (C_B, $\mathrm{ml\,kg^{-1}}$) of any substance (X) was calculated taking the concentration of the substance in the water $[X]_w$ corrected for fish body mass (M) and plasma concentration $[X]_p$ and calculated as:

$$C_{B,X} = [X]_w V_f / M[X]_p, \quad (3)$$

where V_f is the volume of water surrounding the fish. A pulse was identified as a sudden increase in urea, acetamide or thiourea appearance in the surrounding water of at least $20\,\mu\mathrm{mol\,kg^{-1}}$. The branchial permeability (P, $\mathrm{cm\,s^{-1}}$) of any substance (X) was determined as:

$$P_X = C_{B,X}/T \times SA_B \quad (4)$$

using the calculated values of branchial clearance (C_B) and branchial surface area for toadfish (SA_B) ($1920\,\mathrm{cm^2\,kg^{-1}}$; Hughes and Grey, 1972), where T is the duration of the sampling period.

All the following rates were related to fish body mass by expressing urinary flow rate (UFR) in $\mathrm{ml\,kg^{-1}\,h^{-1}}$. Urinary excretion rates (U) of any substance (X) were calculated as:

$$U_X = [X]_u \mathrm{UFR} \quad (5)$$

using measured values of urine flow rate (UFR) and urine concentration $[X]_u$.

Glomerular filtration rates (GFR) were calculated as the clearance of [^{3}H]PEG 4000, i.e. the excretion of radioactivity in the urine ($\mathrm{cpm_u}$) relative to its concentration in the blood plasma ($\mathrm{cpm_p}$):

$$\mathrm{GFR} = \mathrm{cpm_u UFR}/\mathrm{cpm_p}. \quad (6)$$

The filtration rate (FR) of a substance X at the glomeruli was calculated as:

$$FR_X = [X]_p \mathrm{GFR}, \quad (7)$$

and, consequently, the tubular secretion rate (TS) of X was calculated as:

$$TS_X = U_X - FR_X. \quad (8)$$

The renal clearance rate by secretion (CR) of X was calculated as:

$$CR_X = TS_X/[X]_p. \quad (9)$$

Statistical analyses

Data are reported as means ±1 S.E.M. (N is the number of fish). Regression lines were fitted using the method of least squares, and the significance (P<0.05) of the Pearson's correlation coefficient r was assessed. The significance of differences between means was evaluated using Student's paired or unpaired, two-tailed or one-tailed t-tests (P<0.05) as appropriate (Nemenyi et al., 1977).

Table 1. *The pattern of brachial urea excretion in gulf toadfish prior to and following treatment with the urea analogues acetamide or thiourea*

	Control	Analogue treatment
Acetamide series (N=5)		
Urea excretion rate ($\mu\mathrm{mol\,kg^{-1}\,h^{-1}}$)	67.5±16.3	52.4±7.3
Pulse frequency (pulses $12\,\mathrm{h^{-1}}$)	1.20±0.20	0.95±0.35
Pulse size ($\mu\mathrm{mol\,urea\,kg^{-1}\,pulse^{-1}}$)	664±172	606±79
Thiourea series (N=5)		
Urea excretion rate ($\mu\mathrm{mol\,kg^{-1}\,h^{-1}}$)	47.7±11.4	20.2±2.0*,†
Pulse frequency (pulses $12\,\mathrm{h^{-1}}$)	1.20±0.37	0.50±0.11*
Pulse size ($\mu\mathrm{mol\,urea\,kg^{-1}\,pulse^{-1}}$)	510±124	512±71

Values are means ± 1 S.E.M.
*Significantly different (P<0.05) from the pre-analogue control.
†Significantly different (P<0.05) from the acetamide series.

Results

Branchial excretion

The toadfish used in this study were predominantly ureotelic, excreting 80 % urea nitrogen (urea-N; 81.7±11.1 $\mu\mathrm{mol\text{-}N\,kg^{-1}\,h^{-1}}$) and 20 % ammonia-N (23.2±3.3 $\mu\mathrm{mol\text{-}N\,kg^{-1}\,h^{-1}}$) when averaged over the experimental period of 60 h. The gills were the major route of urea excretion, averaging 56.9±9.9 $\mu\mathrm{mol\,kg^{-1}\,h^{-1}}$ (N=10) relative to a urinary excretion rate of only 1.91±0.07 $\mu\mathrm{mol\,kg^{-1}\,h^{-1}}$ (N=10). More than 90 % (92.5±2.4 %, N=10) of the branchial excretion occurred in discrete pulses, with a mean frequency of 1.20±0.20 pulses $12\,\mathrm{h^{-1}}$ (N=10) and a mean size of 596±107 $\mu\mathrm{mol\,kg^{-1}\,pulse^{-1}}$ (N=10) during the 12 h control period. The permeability of the gills to urea was significantly greater during pulse events (7.79×10^{-6}±$1.68\times10^{-6}\,\mathrm{cm\,s^{-1}}$, N=10; minimum estimate, see Discussion) than during non-pulsing periods (0.26×10^{-6}±$0.10\times10^{-6}\,\mathrm{cm\,s^{-1}}$, N=10). Loading the fish with acetamide had no effect on these patterns, but loading with thiourea caused a greater than 50 % reduction in branchial urea excretion rate (Table 1). This effect was entirely due to a significant reduction in pulse frequency; mean pulse size was not affected (Table 1).

Overall, 94.8±0.1 % (N=5) of acetamide excretion occurred through the gills and 72.9±12.6 % (N=5) of this occurred in pulse events. Pulses of acetamide excretion through the gills always occurred simultaneously with those of urea (18 pulse events in five fish over 48 h; e.g. Fig. 1A), and there was a strong quantitative correlation between acetamide and urea pulses. When expressed as plasma clearance ($\mathrm{ml\,kg^{-1}}$), acetamide clearance exhibited a linear proportional relationship (r=0.85, P<0.001) to urea clearance during pulses with a slope of 0.35 and an intercept not significantly different

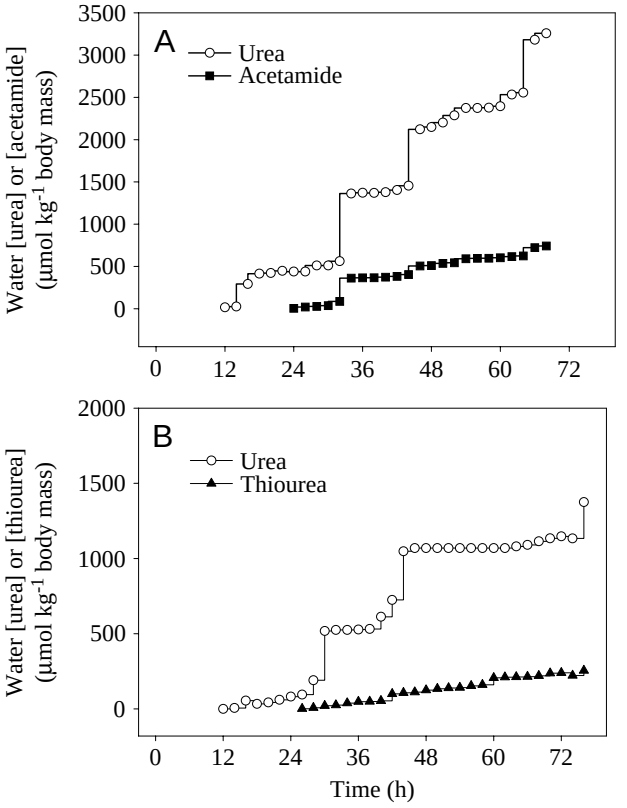

Fig. 1. Data from two individual fish showing the appearance of (A) acetamide and urea and (B) thiourea and urea in the surrounding water over time. Branchial pulsatile excretion of both urea and acetamide is evident. In contrast, thiourea pulses are poorly defined. Values are corrected for fish body mass.

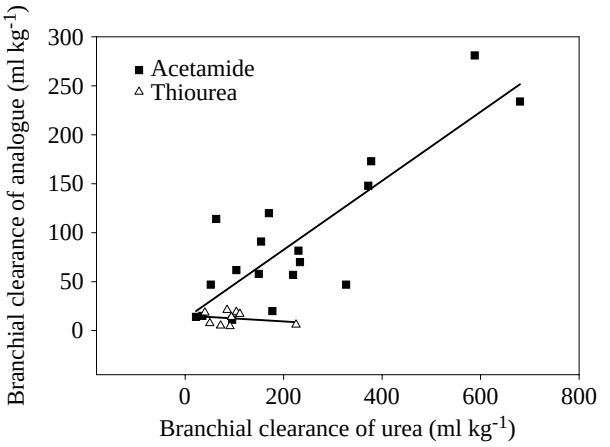

Fig. 2. Linear regressions of the branchial clearance of analogue (*y*-axis) *versus* the branchial clearance of urea (*x*-axis) indicating a clearance of analogue that is less than that of urea. The equation for the acetamide line and the significance (P<0.05) of the correlation is y=0.352x+12.089, r^2=0.724 (N=18), showing that an acetamide pulse is 35 % of the size of a urea pulse. The equation of the thiourea line and the significance (P>0.05) of the correlation is y=–0.030x+15.359, r^2=0.058 (N=9), demonstrating that thiourea clearance is not strongly correlated with urea clearance and that thiourea is poorly cleared by the gills.

from zero (Fig. 2). When expressed as clearance ratio for individual pulses (acetamide/urea), the ratio was 0.50±0.09 (N=18). Thus, branchial acetamide clearance during pulses was 50–65 % less than urea clearance. The permeability of the gills to acetamide during pulse events was 18 times greater than during non-pulsing periods, but was approximately 55 % less than the permeability of the gills to urea during the same pulse events (Table 2).

The relationship between thiourea excretion and urea excretion was much weaker. Only 63.8±0.1 % (N=5) of thiourea excretion occurred through the gills, and 56.2±10.5 % (N=5) of this occurred in pulse events. Pulses of thiourea excretion occurred simultaneously with those of urea excretion (nine pulse events in five fish over 48 h; e.g. Fig. 1B), but there were two additional thiourea pulses that did not appear to be associated with urea pulses. Furthermore, when expressed as plasma clearances, there appeared to be no quantitative relationship between thiourea and urea pulses (r=–0.19; slope not significantly different from zero; Fig. 1B). Note that urea clearances occurred over an overall lower range in the thiourea-treated fish, reflecting higher plasma urea concentrations than in the acetamide-treated fish, despite similar absolute urea pulse sizes. When expressed as clearance ratio for individual pulses (thiourea/urea), the ratio was 0.16±0.04 (N=9), significantly lower than the value for acetamide (see above). Thus, thiourea clearance during pulses was at best only 16 % of the clearance of urea. The permeability of the gills to thiourea was five times greater during pulse events than during non-pulsing periods, but was approximately ninefold less than the permeability of the gills to urea during these same pulse events (Table 2). Thus, substantial differences in permeability (urea>acetamide>thiourea) were seen during pulse events, whereas all three analogues exhibited similar low permeabilities during non-pulsing periods.

Table 2. *Gill permeability to urea, acetamide and thiourea during non-pulsing and pulsing periods*

	Urea permeability ($cm\,s^{-1}$)	Analogue permeability ($cm\,s^{-1}$)
Acetamide series (N=5)		
Non-pulsing	$0.41\times10^{-6}\pm0.17\times10^{-6}$	$0.27\times10^{-6}\pm0.10\times10^{-6}$
Pulsing	$10.48\times10^{-6}\pm2.66\times10^{-6}$*,†	$4.71\times10^{-6}\pm1.01\times10^{-6}$*
Thiourea series (N=5)		
Non-pulsing	$0.11\times10^{-6}\pm0.04\times10^{-6}$	$0.10\times10^{-6}\pm0.02\times10^{-6}$
Pulsing	$5.10\times10^{-6}\pm1.45\times10^{-6}$*,†	$0.56\times10^{-6}\pm0.07\times10^{-6}$*

Values are means ± 1 S.E.M.
*Significantly different (P<0.05) from non-pulsing permeability.
†Significantly different (P<0.05) from analogue permeability during the same period.

Urinary excretion

Our ureteral catheterization technique was designed to avoid modification of the urine by residence in the bladder. Nevertheless, the concentration of urea in ureteral urine (7.96±0.36 mmol l^{-1}, *N*=10) was not significantly different from that in bladder urine (8.60±0.55 mmol l^{-1}, *N*=72) collected from a separate set of fish (see Materials and methods). Notably, both bladder urine and ureteral urine urea concentrations were much greater than plasma urea concentrations (4.47±0.59 mmol l^{-1}, *N*=10). Ion levels were lower in bladder urine (Na^+, 11.9±2.0 mmol l^{-1}, *N*=39; Cl^-, 80.7±4.4 mmol l^{-1}, *N*=59) compared with concentrations in urine that had only been modified by the kidneys (Na^+, 27.9±4.8 mmol l^{-1}, *N*=10; Cl^-, 106.0±10.7 mmol l^{-1}, *N*=10). The plasma level of Na^+ was 154.0±2.9 mmol l^{-1} (*N*=10), and the plasma level of Cl^- was 126.4±2.8 mmol l^{-1} (*N*=10).

On the basis of the renal clearance of the glomerular filtration rate marker [^{3}H]PEG 4000, the toadfish had a glomerular filtration rate (GFR) that was significantly greater than zero (0.05±0.01 ml kg^{-1} h^{-1}, *N*=10), suggesting the presence of a small number of functional glomeruli. However, the urine flow rate (UFR; 0.30±0.11 ml kg^{-1} h^{-1}, *N*=10) was substantially greater than the glomerular filtration rate, indicating that the urine was largely formed by secretion. Consistent with the relationship between UFR and GFR was a net secretion of urea (1.56±0.46 μmol kg^{-1} h^{-1}, *N*=10) that was approximately 10 times greater than its net filtration (0.15±0.04 μmol kg^{-1} h^{-1}, *N*=10).

Fish treated with acetamide demonstrated relatively stable urine urea concentrations (mean 8.35±0.61 mmol l^{-1}, *N*=5) that were significantly greater than plasma concentrations (mean 3.37±0.06 mmol l^{-1}, *N*=5) throughout the experimental period (Fig. 3A). Plasma and urine acetamide concentrations were not significantly different from one another (means 3.48±1.17 mmol l^{-1}, *N*=5 and 2.52±0.77 mmol l^{-1}, *N*=5, respectively) and had a tendency to decrease throughout the experiment as acetamide was cleared from the gills (Fig. 3B). If anything, acetamide levels were greater in the plasma than in the urine, although this difference was not significant.

As in the acetamide series, urea concentrations in the urine of fish treated with thiourea (mean 8.84±0.98 mmol l^{-1}, *N*=5) were significantly greater than plasma levels (mean 5.01±0.98 mmol l^{-1}, *N*=5), although urine levels were not as stable as those seen in the acetamide series (Fig. 4A). Thiourea concentrations in the urine and plasma showed the same trend as urea concentrations, in that thiourea concentration was much greater in the urine (mean 12.22±1.68 mmol l^{-1}, *N*=5) than in the plasma (mean 3.81±0.22 mmol l^{-1}, *N*=5), suggesting that the kidney handles thiourea in the same way that it handles urea (Fig. 4B) and very differently from the way it handles acetamide (Fig. 3B).

Fish treated with thiourea exhibited substantially lower secretion clearance rates of urea through the kidney (Fig. 5A,B) than did those treated with acetamide, suggesting that thiourea may have played an inhibitory role at the kidney comparable with that at the gills (Table 1). In simultaneous

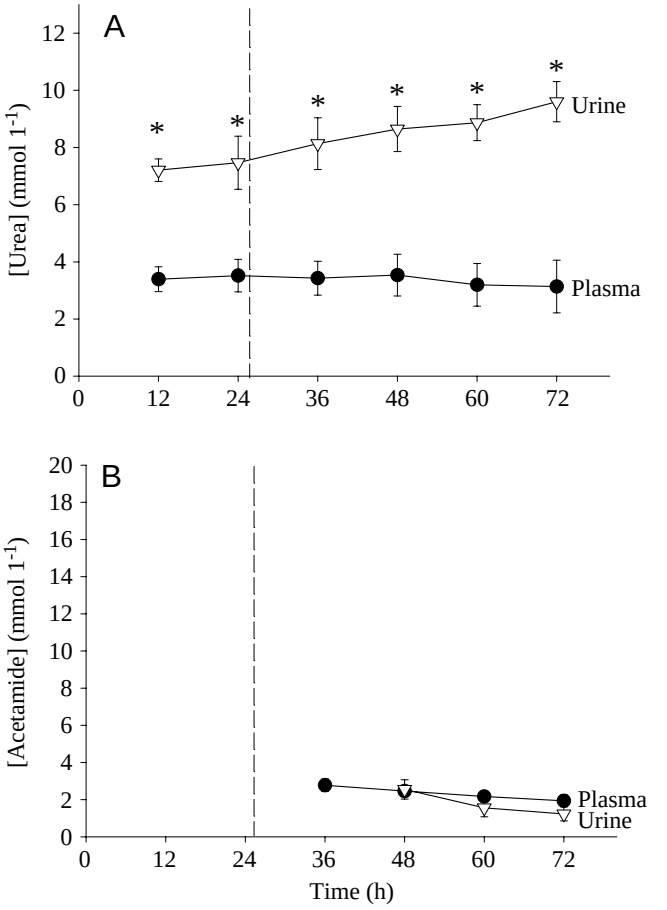

Fig. 3. Concentrations of (A) urea and (B) acetamide in the urine and plasma demonstrating differences in the renal handling of the two substances. The broken line indicates the time of acetamide injection. Values are means ±1 S.E.M. (*N*=5). An asterisk indicates a significant difference (*P*<0.05) compared with the plasma concentration.

secretion clearance measurements, relative urea secretion (0.83±0.29 ml kg^{-1} h^{-1}, *N*=5) was almost four times greater than relative acetamide secretion (0.22±0.18 ml kg^{-1} h^{-1}, *N*=5) (Fig. 5A). In contrast, in the presence of thiourea, the secretion clearance of urea (0.18±0.03 ml kg^{-1} h^{-1}, *N*=5) was less than that of thiourea (0.35±0.02 ml kg^{-1} h^{-1}, *N*=5) (Fig. 5B). When expressed as a clearance ratio (analogue/urea) for all collection periods in which simultaneous measurements were made, the ratios were 1.97±0.24 (*N*=5) for thiourea and only 0.19±0.09 (*N*=5) for acetamide, a highly significant difference.

Plasma Na^+ concentration in both the acetamide and thiourea series was on average seven times greater than urine concentrations (Table 3). In contrast, plasma and urine Cl^- concentrations were not significantly different from one another. When expressed as secretion clearance rates, Cl^- and urea exhibited a linear proportional relationship (*r*=0.800, *P*<0.001) with a slope of 2.70 and an intercept not significantly different from zero (Fig. 6A). An almost identical relationship (slopes not significantly different) was observed when water movement was plotted against urea secretion rates (*r*=0.820, *P*<0.001; slope 2.94). Thus, on a relative basis, 2.7–2.9 times

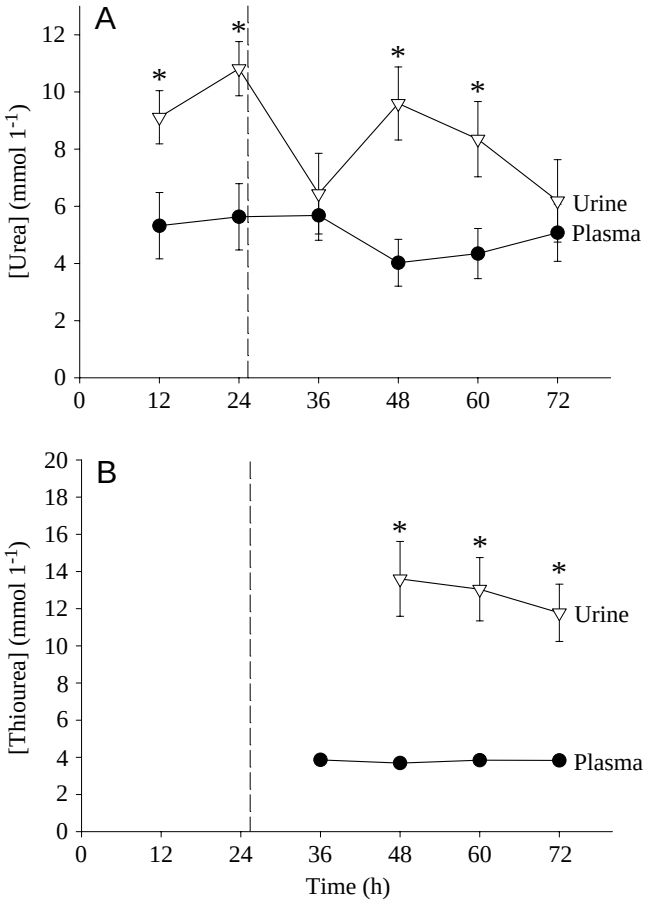

Fig. 4. Concentrations of (A) urea and (B) thiourea in the urine and plasma demonstrating similar renal handling of the two substances. The broken line indicates the time of thiourea injection. Values are means ±1 S.E.M. (N=5). An asterisk indicates a significant difference (P<0.05) compared with the plasma concentration.

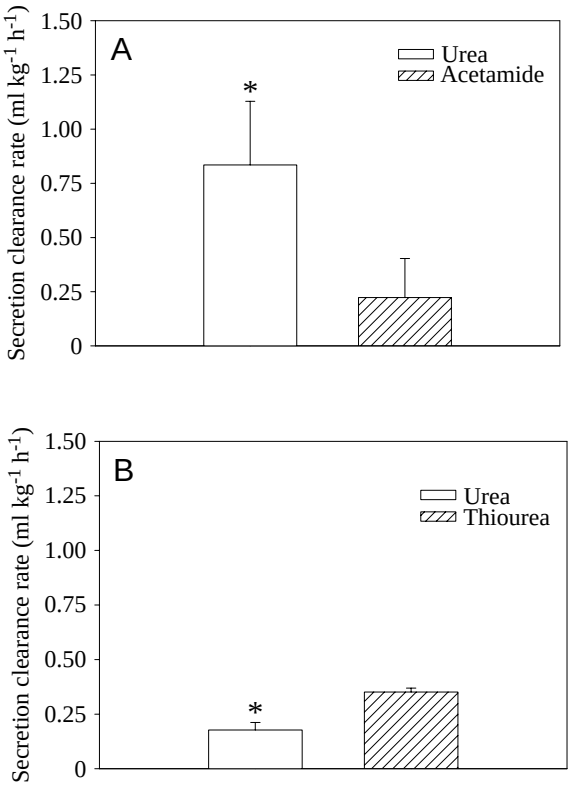

Fig. 5. (A) The secretion clearance rate (ml kg^{-1} h^{-1}) of fish in the acetamide series shows a urea clearance rate that is significantly greater than the clearance rate of acetamide. In contrast (B), the clearance rate of urea is significantly less than that of thiourea. Values are means +1 S.E.M. (N=5 for each treatment). An asterisk indicates a significant difference (P<0.05) compared with the analogue.

more urea was secreted by the kidney than Cl^- or water movement. Predictably, there was no quantitative relationship between the secretion of Na^+ and urea (r=0.344; slope not significantly different from zero; Fig. 6A), which is consistent with the finding that Na^+ was more concentrated in the plasma whereas urea was more concentrated in the urine. Overall, there was no net secretion of Na^+.

The secretion clearance of thiourea exhibited a linear proportional relationship with that of Cl^- (r=0.683, P<0.001) with a slope of 2.08 and an intercept not significantly different from zero (Fig. 6B). A similar relationship (slopes not significantly different) was observed when thiourea secretion was plotted against water movement rates (r=0.797, P<0.001; slope 2.58). Thus, like urea, the renal secretion of thiourea was 2–2.6 times greater than either Cl^- secretion or water movement. Unlike the patterns of secretion observed for both urea and thiourea, the secretion clearance of acetamide exhibited a 1:1 relationship with both Cl^- and water (Cl^-, r=0.963, P<0.001; slope 0.88; water, r=0.962, P<0.001; slope 0.94) (Fig. 6C). Therefore, on a relative basis, acetamide moved into the kidney tubule at the same rate as Cl^- and water.

Table 3. *Na^+ and Cl^- concentrations in the plasma and urine of acetamide- and thiourea-treated fish*

	[Na^+] (mmol l^{-1})		[Cl^-] (mmol l^{-1})	
	Plasma	Urine	Plasma	Urine
Acetamide-treated fish (N=5)	158.1±3.2*	18.7±5.7	127.4±4.7	117.3±14.9
Thiourea-treated fish (N=5)	150.1±4.4*	27.1±5.4	125.9±2.2	93.7±15.1

Values are means ± 1 S.E.M.
*Significantly different (P<0.05) from urine concentrations. There were no significant differences between the two series.

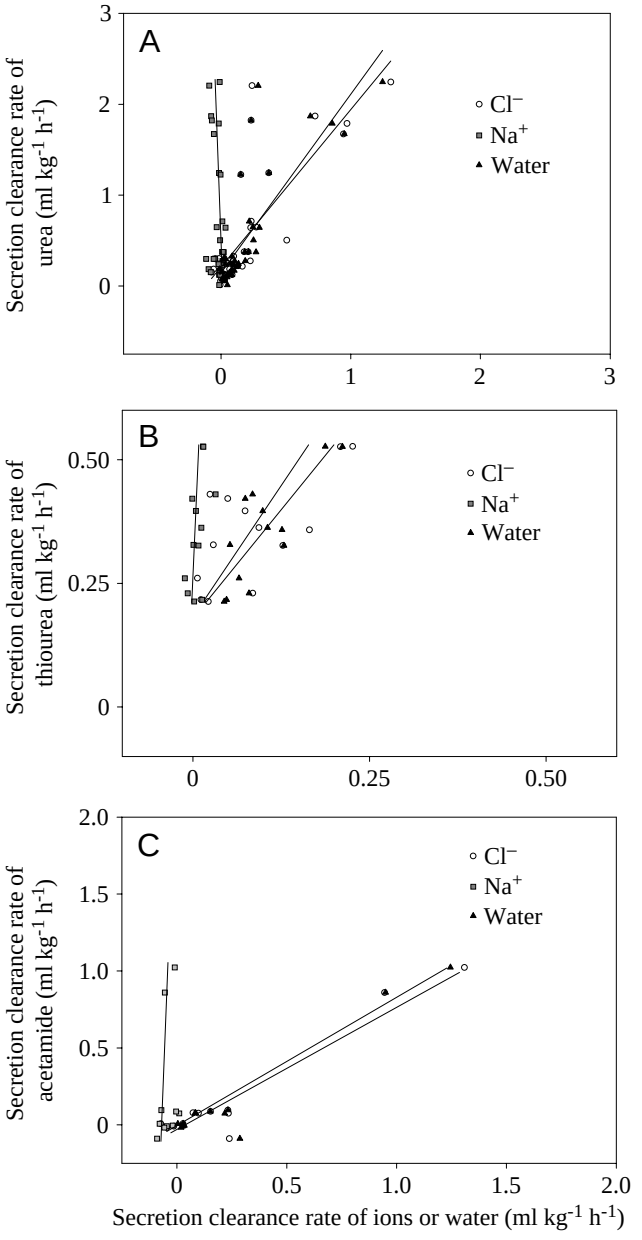

Fig. 6. Linear regressions of the secretion clearance rates of (A) urea, (B) thiourea and (C) acetamide (*y*-axis) *versus* water, Na^+ and Cl^- (*x*-axis) demonstrating a secretion of urea and thiourea at a greater relative rate than the movements of water and Cl^- and a rate of secretion of acetamide comparable with those of both Cl^- and water. In all cases, there was no net secretion of Na^+.

Discussion

The basis of this study was to exploit the differential handling of urea and its analogues by urea transport mechanisms to determine whether a urea transporter was present in the kidney of the gulf toadfish and to characterize further the previously documented facilitated diffusion transport mechanism in the gills (Walsh, 1997; Wood et al., 1998; Smith et al., 1998). Our results suggested that there are two distinct transport mechanisms involved in the excretion of urea in the gulf toadfish, one in the gill and one in the kidney, both of which differentially handle urea, acetamide and thiourea, but with differing capabilities. Notably, acetamide and urea were handled similarly by the gills, with acetamide clearance exhibiting a linear relationship with urea clearance but being 50–65 % less than urea clearance. In contrast, the relationship between thiourea excretion and urea excretion was much weaker, and relative thiourea clearance was at most only 16 % of that of urea. The opposite trend was observed in the kidney, where it appeared that thiourea and urea were handled similarly, accumulating in the urine against a concentration gradient. Conversely, acetamide appeared to equilibrate between the plasma and urine. The secretion of thiourea was equal to or greater than that of urea, whereas that of acetamide was less than 25 % that of urea.

Differences between the handling of urea and analogues amongst organs of the same species is a phenomenon that is not unprecedented. A distinction has also been noted in the handling of urea and analogues by the skin and urinary bladder of the toad *Bufo viridis* (Garcia-Romeu et al., 1981; Shpun and Katz, 1990). Using four times more analogue than urea, thiourea was found to interact competitively, thereby inhibiting urea transport by the facilitated diffusion transport mechanism in the urinary bladder, while acetamide did not have an effect (Shpun and Katz, 1990). In contrast, in the toad skin, acetamide, at five times greater concentrations than urea, inhibited the active transport of urea while thiourea did not interact with the transporter (Garcia-Romeu et al., 1981).

In *Rana esculenta*, the absorption of urea by the skin is believed to be *via* an ion-independent active transporter and, although it has the same analogue transport qualities, it is quite unlike the facilitated diffusion mechanism in the toadfish gill, which is thought to be under AVT control (Gilmour et al., 1998; Perry et al., 1998). However, the urinary bladder of the toad *Bufo viridis*, in which there is believed to be an AVT-sensitive facilitated diffusion mechanism, exhibited an acetamide permeability that was 40 % of that of urea and a thiourea permeability that was only 1 % of that of urea (Levine et al., 1973a,b). This is similar to the present observations on toadfish gills. Thus, although it appears that analogue permeability is a good tool for discriminating between transport mechanisms within one species, one cannot be certain of the type of urea transport mechanism entirely on the basis of the handling of analogues amongst species.

Increases in urea, acetamide and thiourea permeability of the gills were observed during pulsing events, although analogue permeability was significantly lower than that of urea. The present findings on branchial thiourea handling during pulses were quantitatively comparable with those of Wood et al. (1998), who demonstrated a significant, although much reduced, increase in thiourea permeability compared with urea permeability. Despite small differences in calculated oil/water partition coefficients and lipid permeabilities (Goldstein and Solomon, 1960; Lippe, 1969), all three substances had similar permeabilities during non-pulsing periods, suggesting that the gill handled urea, acetamide and thiourea identically when the facilitated diffusion transporter was not activated.

In the present study, thiourea appeared to inhibit the branchial excretion of urea by reducing the pulse frequency rather than the size of the individual events. Studies performed on toad bladder and rat inner medullary collecting ducts have demonstrated the inhibitory effects of both acetamide and thiourea on urea transport, with the latter being more effective in this regard (Levine et al., 1973b; Eggena, 1973; Chou and Knepper, 1989). Chou and Knepper (1989) demonstrated a 74 % inhibition by thiourea and a 35 % inhibition by acetamide at analogue concentrations of 200 mmol l^{-1}. The concentrations of urea and analogue used for that study were greater than the concentrations used in the present study (approximately 4 mmol l^{-1}, which is approximately equal to the plasma urea concentration). Correspondingly, the extent of competitive inhibition in the present study was much less, thiourea inhibiting urea excretion by approximately 50 % and acetamide showing no inhibition of urea transport. Using a high level of thiourea (60 mmol l^{-1}) in the external sea water, Wood et al. (1998) demonstrated a 73 % inhibition of urea influx permeability during pulse events.

The appearance of [^{3}H]PEG 4000 in the urine of the toadfish supports the findings of Lahlou et al. (1969) demonstrating the appearance of [^{14}C]inulin in the urine of the oyster toadfish *Opsanus tau*. In examining whether the radioactivity appearing in the urine was incorporated into inulin, they ascertained that inulin was being degraded into a substance of lower molecular mass, resulting in only one-third of the ^{14}C label in the urine actually being inulin. With everything taken into account, Lahlou et al. (1969) estimated that the true urine-to-plasma (U/P) ratio for inulin was probably 0.1–0.2. PEG 4000 is thought to be a more accurate indicator of GFR than inulin derivatives since it undergoes minimal metabolic breakdown (Beyenbach and Kirschner, 1976; Erickson and Gingerich, 1986; Curtis and Wood, 1991). The U/P ratio for PEG 4000 was approximately 0.19, a value at the high end of the range proposed by Lahlou et al. (1969) for the oyster toadfish. Consequently, the appearance of [^{3}H]PEG 4000 in the urine confirms that there are a small number of functional glomeruli present in the kidney of the gulf toadfish *Opsanus beta* and probably in the oyster toadfish *Opsanus tau* as well.

Our results suggest the presence of a urea transport mechanism in the toadfish kidney that is unlike the facilitated diffusion transporter in the gill (Smith et al., 1998) for two reasons. First, as mentioned above, the analogues are handled differently by the kidney and by the gill, suggesting a unique transporter in the kidney. Second, the transport of thiourea and urea could not occur exclusively *via* a facilitated diffusion mechanism. The renal secretion rate of urea was greater than the rate of secretion of acetamide, although significantly less than the rate of secretion of thiourea. Both thiourea and urea appeared to be secreted at least 2–3 times more effectively than Cl^- and water. Acetamide secretion, however, was well correlated with Cl^- secretion, with the ratio of secretion being 1:1. Both substances were found in equal concentrations in the urine and plasma and thus appeared to equilibrate passively between the two fluids. Since the relative rates of secretion of thiourea and urea were greater than the relative rates of movement of Cl^- or water, their passage into the kidney tubule cannot be accounted for by passive diffusion. The transport of urea and thiourea also occurred against a concentration gradient, supporting the theory of an active transport mechanism.

What transport mechanism could be involved in the secretion of urea into the kidney tubule of the gulf toadfish? While a facilitated diffusion transporter has been cloned from shark kidney (Smith and Wright, 1999), the most studied active transport mechanism is the Na^+-dependent urea transporter originally identified in the elasmobranch kidney (Schmidt-Neilsen et al., 1972) and more recently in the mammalian kidney of protein-depleted animals (Isozaki et al., 1994a,b). One hypothesis for the toadfish kidney is that urea secretion is *via* a Na^+/urea cotransporter, urea moving with Na^+ down its concentration gradient. Further down the tubule, Na^+ would then be actively reabsorbed against its concentration gradient. A second and simpler hypothesis is a Na^+/urea antiporter, for which evidence has recently been provided in the deep portions of the inner medullary collecting duct ($IMCD_3$) of the mammalian kidney; urea appears to move into the kidney tubule in direct countertransport with active Na^+ reabsorption (Kato and Sands, 1998a,b). This Na^+/urea antiport mechanism appears to have the characteristics of two different transporters. It is similar both to the facilitated diffusion mechanism (UT-A1), in that it is stimulated by arginine vasopressin (AVP) and inhibited by phloretin, and to the Na^+-coupled transporter, in that it is inhibited by ouabain and an absence of Na^+ on the mucosal side (Kato and Sands, 1998a).

Further investigation is needed to establish whether urea secretion in the kidney of the toadfish is an active transport mechanism that relies on the movement of Na^+ or, conceivably, a unique mechanism not yet observed in other systems.

This study was supported by an NSF grant (IBN-9507239) to P.J.W. and an NSERC research grant to C.M.W.

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